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Regulation of the Glutamate/glutamine Cycle by Nitric Oxide in the Central Nervous System

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Abstract

Nitric oxide (NO) is a critical contributor to glutamatergic neurotransmission in the central nervous system (CNS). Much of its influence is due to the ability of this molecule to regulate protein structure and function through its posttranslational modification of cysteine residues, a process known as S-nitrosylation. However, little is known about the extent of this modification and its associated functional effects in the brain under physiological conditions. We employed mass spectrometry (MS)-based methodologies to interrogate the S-nitrosocysteine proteome in wild-type (WT), neuronal nitric oxide synthase-deficient (nNOS $^{-/-}$), and endothelial nitric oxide synthase-deficient (eNOS $^{-/-}$) mouse brain. These approaches identified 269 sites from 136 proteins in the WT brain, with notable reductions in the number of sites detected in either eNOS $^{-/-}$ (50% of WT) or nNOS $^{-/-}$ brain (26% of WT). Gene ontology analysis revealed a cluster of S-nitrosylated proteins participating in the glutamate/glutamine cycle in wild-type and eNOS $^{-/-}$ mice that was underrepresented in nNOS $^{-/-}$ animals, suggesting a role for nNOS-derived NO in the regulation of glutamate utilization in the CNS. Functional profiling of this pathway using ^{15}N -glutamine based metabolomic analyses and enzymatic activity assays uncovered decreased conversion of glutamate to glutamine and increased glutamate oxidation in nNOS $^{-/-}$ mice relative to the other genotypes. Furthermore, site-directed mutagenesis of the rat sodium-dependent excitatory amino acid transporter 2 at Cys373 and Cys562 (Cys561 in mouse sequence), the two sites of S-nitrosylation observed in wild-type and eNOS $^{-/-}$ mice, revealed inhibition of glutamate transport through reversible S-nitrosylation. The selective, nNOS-dependent S-nitrosylation of proteins that govern glutamate transport and metabolism identifies a previously unknown function for NO in glutamatergic neurotransmission.

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REGULATION OF THE GLUTAMATE/GLUTAMINE CYCLE BY NITRIC OXIDE IN THE
CENTRAL NERVOUS SYSTEM

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“Science is fun. Science is curiosity. We all have natural curiosity. Science is a process of investigating. It’s posing questions and coming up with a method. It’s delving in.”

- Sally Ride

To my family, who always supported my curiosity (even when it was inconvenient).

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ABSTRACT

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Karthik Anderson Raju

Harry Ischiropoulos

Nitric oxide (NO) is a critical contributor to glutamatergic neurotransmission in the central nervous system (CNS). Much of its influence is due to the ability of this molecule to regulate protein structure and function through its posttranslational modification of cysteine residues, a process known as S-nitrosylation. However, little is known about the extent of this modification and its associated functional effects in the brain under physiological conditions. We employed mass spectrometry (MS)-based methodologies to interrogate the S-nitrosocysteine proteome in wild-type (WT), neuronal nitric oxide synthase-deficient ($\text{nNOS}^{-/-}$), and endothelial nitric oxide synthase-deficient ($\text{eNOS}^{-/-}$) mouse brain. These approaches identified 269 sites from 136 proteins in the WT brain, with notable reductions in the number of sites detected in either $\text{eNOS}^{-/-}$ (50% of WT) or $\text{nNOS}^{-/-}$ brain (26% of WT). Gene ontology analysis revealed a cluster of S-nitrosylated proteins participating in the glutamate/glutamine cycle in wild-type and $\text{eNOS}^{-/-}$ mice that was underrepresented in $\text{nNOS}^{-/-}$ animals, suggesting a role for nNOS-derived NO in the regulation of glutamate utilization in the CNS. Functional profiling of this pathway using ^{15}N -glutamine based metabolomic analyses and enzymatic activity assays uncovered decreased conversion of glutamate to glutamine and increased glutamate oxidation in $\text{nNOS}^{-/-}$ mice relative to the other genotypes. Furthermore, site-directed mutagenesis of the rat sodium-dependent excitatory amino acid transporter 2 at Cys³⁷³ and Cys⁵⁶² (Cys⁵⁶¹ in mouse sequence), the two sites of S-nitrosylation observed in wild-type and $\text{eNOS}^{-/-}$ mice, revealed inhibition of glutamate transport through reversible S-nitrosylation. The selective, nNOS-dependent S-nitrosylation of

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CHAPTER 1

Background

1.1 Introduction

Since its initial discovery and characterization in the 1980s, nitric oxide (NO) has become widely accepted as a critical signaling molecule in biological function. Indeed, the field of NO biology owes much to the pioneering work of Furchgott, Ignarro, Murad, Moncada, and Hibbs, among others who identified roles for NO in vasodilation and immune responses to bacterial infection. The significance of this research was further emphasized by the 1998 Nobel Prize in Medicine and Physiology, which recognized NO as a vital signaling intermediate in the cardiovascular system (though it was appreciated in other systems as well). NO was initially thought to achieve its effects by binding to the heme iron of soluble guanylate cyclase (sGC), resulting in the increased conversion of guanosine-5'-triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). The ensuing activation of downstream cGMP-dependent signaling cascades ultimately lead to physiological changes, such as smooth muscle relaxation. However, an alternative mechanism of NO -based regulation was discovered in the early 1990s, in which NO could form stable adducts with protein cysteine residues to produce S-nitrosocysteine. The generation of such protein S-nitrosocysteines was also found to result in physiological changes, including vasodilation and platelet inhibition¹. Subsequent studies have reinforced the importance of this posttranslational modification (known as S-nitrosylation) as an effector of NO -dependent signaling².

Since that initial study in 1992, over 1100 S-nitrosocysteine residues have been identified in more than 640 proteins across several cell and organ systems³⁻⁶. These proteins are involved in a wide array of cellular processes, with S-nitrosylation implicated as a direct regulator of protein function and subcellular localization in multiple targets⁷⁻⁹. In particular, its physiological and pathophysiological contributions to central nervous system (CNS) function have elicited intense interest. Several studies have investigated the regulatory roles of S-nitrosylation on specific proteins involved in neurogenesis¹⁰, synaptic transmission¹¹, and neurodegeneration¹². However, such rigid focus on specific targets has led to broader questions concerning the global coordination of cellular processes by S-nitrosylation in the brain, especially under physiological conditions. By employing an array of methodologies, ranging from mass spectrometry (MS)-based proteomic identification of S-nitrosoproteins to more conventional molecular biology and enzymatic approaches, one can start to answer at least some of these questions. In doing so, a greater understanding of $\cdot\text{NO}$'s role in both CNS development and dysfunction can be achieved, while also providing valuable preliminary information for future studies.

1.2 Chemical Properties of Nitric Oxide

Nitric oxide (also known as nitrogen monoxide) is a gas that was first described in 1774 by Joseph Priestly in his *Experiments and Observations on Different Kinds of Air, Vol. 1*. A diatomic free radical, $\cdot\text{NO}$ consists of one nitrogen and one oxygen atom, with an unpaired valence electron. Due to both

its low solubility in water¹³ (1.9 mM at 20°C) and liposome/water partition coefficient¹⁴ of 4.4, $\cdot\text{NO}$ can diffuse readily through hydrophobic environments (including the plasma membrane). Additionally, it can react with several targets to generate a variety of end products¹⁵.

The reactive nature of $\cdot\text{NO}$ contributes to variations in its half-life, particularly through its interactions with oxygen species. In aqueous solution, $\cdot\text{NO}$ reacts with O_2 and possesses a half-life inversely proportional to its concentration¹⁶⁻¹⁷. In plasma and other physiological buffers, $\cdot\text{NO}$ oxidation to both nitrite (NO_2^-) and nitrate (NO_3^-) is facilitated by hemoglobin and other proteins through heme and/or other metal chemistries. The resulting $\cdot\text{NO}$ metabolites possess an extended half-life¹⁸⁻¹⁹. Furthermore, nitric oxide can react with the free radical superoxide (O_2^-) to produce the oxidant peroxynitrite (ONOO^-), a reaction that is essentially diffusion-rate limited²⁰.

Metals and lipid-derived radicals can also act as targets of nitric oxide. Metal nitrosyl (M-NO) complexes are readily formed through the coordination of nitric oxide to transition metals such as iron and copper²¹⁻²³, and are important in the regulation of a number of enzymes²⁴⁻²⁵. Lipid peroxidation is also inhibited by the interaction of $\cdot\text{NO}$ with lipid peroxyl radicals, which can occur at a rate near the diffusion limit²⁶⁻²⁸.

Taken together, evidence suggests that the biological availability of $\cdot\text{NO}$ (especially as a signaling molecule) is highly dependent on its local environment.

Given this information, it is necessary to understand three principles about nitric oxide *in vivo*: how, when, and where it is produced. Fortunately, these questions were answered by a number of landmark studies performed between the 1980s and 1990s.

1.3 Biosynthesis of Nitric Oxide

The endogenous synthesis of NO was first proposed in a study from 1916, which noted a higher nitrate and nitrite concentration in human urine than could be accounted for by dietary intake alone²⁹. This finding was further confirmed by isotopic balance experiments utilizing $^{15}\text{NO}_3^-$, which revealed that endogenous production of nitrate (in this case, without ^{15}N) was the source of excess nitrate in urine³⁰. However, the source of these metabolites remained elusive until 1987, when the precursor to both nitrate and nitrite was demonstrated to be L-arginine³¹⁻³². Specifically, ^{15}N -labeling studies found that the nitrogen in both NO_2^- and NO_3^- was derived from one of the two guanidino nitrogens in L-arginine, while ^{14}C -labeling studies identified L-citrulline as the other product³². This mechanism was further characterized in later reports, with converging lines of evidence suggesting NO as an intermediate in the formation of nitrate and nitrite³³⁻³⁴.

Nitric oxide is generated from L-arginine and oxygen, with L-citrulline produced as a by-product. The reaction is catalyzed by three isoforms of the enzyme nitric oxide synthase (NOS): NOS1 (neuronal NOS: nNOS), NOS2

(inducible NOS: iNOS), and NOS3 (endothelial NOS: eNOS). Of the three isoforms, nNOS was the first to be purified and cloned³⁵⁻³⁶, with iNOS³⁷ and eNOS³⁸⁻⁴⁰ following soon afterwards. Each isoform is a homodimer assembly of two 110-150 kDa monomers, composed of an N-terminal oxygenase domain and a C-terminal reductase domain linked by a calmodulin recognition site⁴¹⁻⁴². The oxygenase domain includes binding sites for the cofactors tetrahydrobiopterin (BH₄) and iron protoporphyrin IX, as well as a binding site for the substrate L-arginine. The reductase domain contains binding sites for electron carriers flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), as well as a site for the electron donor NADPH (the reduced form of nicotinamide adenine dinucleotide phosphate). Electrons supplied by NADPH are transported across the reductase domain by FAD and FMN to the heme group of iron protoporphyrin IX in the oxygenase domain, where they can oxidize L-arginine. One of the guanidino nitrogens in L-arginine undergoes a 5-electron oxidation through two mono-oxygenation steps: the first producing *N*^ω-hydroxyarginine (NOHA), which is then oxidized to produce L-citrulline and [•]NO⁴³⁻⁴⁴. Calmodulin binding at its cognate recognition site is thought to stabilize electron transfer between the reductase FMN and the oxygenase heme⁴⁵.

All three NOS isoforms are expressed in the brain, and catalyze the production of [•]NO from L-arginine and oxygen. However, they differ in several key respects, including functional regulation and levels of [•]NO production. Both nNOS and eNOS are constitutively-expressed NOS isoforms and rely on calcium

(Ca²⁺) to initiate transient bursts of \cdot NO synthesis: elevation of intracellular Ca²⁺ stimulates increased binding of calmodulin to both NOS isoforms, leading to the generation of \cdot NO⁴⁶⁻⁴⁸. The function of both isoforms is also subject to regulation by multiple posttranslational modifications (PTMs), including palmitoylation, phosphorylation, S-glutathionylation, and S-nitrosylation⁴⁹⁻⁵². In contrast, iNOS activity is less sensitive to changes in intracellular Ca²⁺, since the enzyme is far more tightly bound to calmodulin than either nNOS or eNOS^{37, 53}. Expression of this isoform is induced by inflammatory stimuli and results in prolonged periods of \cdot NO synthesis⁵⁴⁻⁵⁵. Much less is known regarding its functionally relevant PTMs, though it has been found to be S-nitrosylated⁵⁶. Of the three isoforms, iNOS is thought to generate the highest amounts of \cdot NO, with both nNOS and eNOS producing orders of magnitude less⁵⁷⁻⁵⁸.

In addition to the dissimilarities already outlined, the NOS isoforms also differ in sequence conservation and localization in the brain. Analysis of primary amino acid sequences for the human NOS enzymes revealed only 51-60% homology between the isoforms⁵⁹. However, greater sequence homology for specific isoforms was conserved between multiple species (>80% for iNOS between mouse, rat, dog, and human), suggesting that \cdot NO synthesis may be of importance in mammalian evolution⁶⁰. Given the functional and structural diversity of the NOS isoforms, it is interesting to note their restricted expression patterns in distinct cell types in the brain. nNOS is primarily expressed in defined populations of neurons in the CNS, and associates with specific postsynaptic

complexes due to its PDZ domain⁶¹⁻⁶². iNOS, on the other hand, is mainly expressed in the cytoplasm of astrocytes and microglia during inflammation⁶³⁻⁶⁴. Finally, eNOS is chiefly expressed in the cytoplasm of both astrocytes and vascular endothelial cells in the brain⁶⁵⁻⁶⁷.

1.4 Nitric Oxide in Physiology

1.4.1 Identification of Endothelial Nitric Oxide Synthesis

The long journey in characterizing endogenous biological synthesis of NO began in the latter half of the 17th century, with the discoveries of amyl nitrite and nitroglycerin as potent vasodilators⁶⁸⁻⁷⁰. Despite these early studies, the actual mechanism of action for these molecules (through the release of NO or NO equivalent molecules) remained unknown for close to a century. In 1977, Ferid Murad and colleagues realized that nitrite-containing compounds (including nitroglycerin) and NO gas had similar effects in stimulating sGC activity and subsequent vascular dilation⁷¹, a finding reaffirmed in 1979 by Louis Ignarro's group⁷². Robert Furchgott and John Zawadzki made a parallel observation on vasodilation in 1980, demonstrating that endothelial cells contributed toward the relaxation of smooth muscle⁷³. By 1982, Furchgott's team speculated that this action was achieved through release of an unstable relaxing substance, referred to as endothelial relaxing factor⁷⁴ (EDRF). Four years later, both Furchgott and Ignarro presented independent findings at a vasodilation symposium suggesting that EDRF was actually NO . In the following year, this hypothesis was confirmed by additional findings from Salvador Moncada's group, who found that

endothelium-produced NO derived from L-arginine accounted for EDRF's effects⁷⁵⁻⁷⁶. Ultimately, the source of endothelial NO was shown to be eNOS³⁸⁻⁴⁰. Recognition of NO 's importance in vasodilation came in 1998, with the awarding of the 1998 Nobel Prize in Medicine and Physiology to Furchgott, Ignarro, and Murad.

In the twenty years since those initial studies, multiple biological processes have been found to be regulated by eNOS-derived NO . Mice with a genetic deletion of eNOS (eNOS^{-/-}) exhibit hypertension, hypercoagulability, and increased proliferation of vascular smooth muscle cells after vessel injury⁷⁷⁻⁷⁹. Additionally, they demonstrate greater leukocyte/endothelium interaction, increased infarct size after middle cerebral artery occlusion (MCAO), and decreased beta-oxidation of fatty acids through very long chain acyl-CoA dehydrogenase^{8, 80-81}. Modulation of eNOS activity through phosphorylation at serine 1176 has been shown to affect insulin sensitivity, regulation of insulin levels, adiposity, and vascular reactivity⁸²⁻⁸³. Considered as a whole, the data implicates eNOS-derived NO in the global coordination of vascular and metabolic function.

1.4.2 Nitric Oxide Synthesis and the Immune Response

Prior to Moncada's findings, John Hibbs and colleagues identified L-arginine as an essential substrate for nitrite synthesis following macrophage activation³¹. In doing so, they provided one of the first examples for endogenous

biosynthesis of nitric oxide (though this was not recognized until later). Metabolism of L-arginine by macrophages was necessary for their cytotoxic activity against target tumor cells⁸⁴. However, the generation of $\cdot\text{NO}$ as an intermediate in this process was not established until a series of studies published between 1988 and 1989^{33, 85-86}. Consequently, the involvement of $\cdot\text{NO}$ in several inflammatory cascades has been described⁸⁷⁻⁹⁰. Nitric oxide has been shown to decrease cellular respiration through disruption of iron-sulfur clusters in the electron transport chain⁹¹. Moreover, it can affect membrane potential maintenance, DNA repair, lipid peroxidation, and other critical functions in target cells⁹²⁻⁹⁴.

Eventually, the chief source of $\cdot\text{NO}$ in macrophages and other immune cells was found to be iNOS³⁷, which is upregulated in response to inflammatory cytokines and other stimuli. Mice with a genetic deletion of iNOS (iNOS^{-/-}) are phenotypically similar to wild-type (WT) mice under normal conditions. However, they are more susceptible to parasitic infection, bacterial proliferation, and intestinal tumorigenesis⁹⁵⁻⁹⁸. Concomitantly, they are resistant to sepsis-induced hypotension, inflammation-induced osteoporosis, and ischemia-induced neurodegeneration in the brain^{96, 99-101}. Taken together, the evidence suggests iNOS-derived $\cdot\text{NO}$ as a local inducible regulator of the immune response to pathogens and cellular damage.

1.4.3 Nitric Oxide and the Central Nervous System

Given its systemic nature as a signaling molecule, it is no surprise that nitric oxide also influences brain physiology. Early studies in the cerebellum implicated NO synthesis as an intermediate step between N-methyl-D-aspartate (NMDA) receptor stimulation by glutamate and cGMP synthesis¹⁰²⁻¹⁰³. In doing so, they provided a first glimpse into nitric oxide's role in neurotransmitter signal transduction. These findings were later followed up by multiple observations of NO 's contribution to long-term potentiation (LTP), a measure of synaptic plasticity¹⁰⁴⁻¹⁰⁸.

Subsequent investigations have identified nitric oxide as a critical participant in several biological processes in the CNS. Presynaptic vesicular release and recycling is potentiated by nitric oxide through increased SNARE complex assembly and regulation of phosphatidylinositol 4, 5-bisphosphate (PIP₂)-dependent processes¹⁰⁹⁻¹¹¹. Exocytotic coupling and glutamate/GABA release during neurotransmission is facilitated by NO -based activation of presynaptic hyperpolarization-activated cyclic nucleotide-gated (HCN) channels and protein kinase G¹¹²⁻¹¹⁶ (PKG). Similarly, presynaptic remodeling is accomplished through NO -dependent kainate receptor recruitment and activity-dependent synaptogenesis¹¹⁷⁻¹¹⁹. On the postsynaptic side, NO has been shown to modulate target neuron excitability through regulation of Kv2/Kv3 potassium channel activity and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor trafficking¹²⁰⁻¹²². Finally, NO -dependent upregulation of

glycolysis in astrocytes has been shown to be important in supporting changes in neuronal activity¹²³⁻¹²⁴.

Much of the work documenting NO 's effects on the CNS was only made possible by the initial purification and characterization of nNOS, the first NOS isoform to be isolated³⁵⁻³⁶. In particular, discovery of postsynaptic tethering between the NMDA receptor, PSD-95, and nNOS provided a molecular explanation for the association between synaptic transmission and NO production/activity^{62, 125}. Upon presynaptic release of glutamate into the synaptic cleft, it is free to bind to the postsynaptic NMDA receptor (along with a receptor co-agonist, either glycine or D-serine). Once activated, the NMDA receptor allows Ca^{2+} to enter the neuron. Due to the close proximity of the NMDA receptor to nNOS afforded by the PSD-95 scaffold, this Ca^{2+} influx subsequently leads to NO synthesis.

Predictably, loss of nNOS has profound effects on CNS function. Mice with a genetic deletion of this isoform (nNOS^{-/-}) demonstrate substantially decreased NO production (>90%) relative to WT mice¹²⁶. Consequently, they exhibit a number of phenotypic deficits, including impairments in various forms of recognition, fear conditioning, working memory, and other cognitive functions¹²⁷⁻¹³⁰. Many, if not all, of these insufficiencies may be symptomatic of widespread synaptic dysfunction across the brain: several reports have documented decreases in both synaptic potentiation and depression in these mice relative to WT animals¹³¹⁻¹³². Correspondingly, nNOS^{-/-} mice also demonstrate an

attenuated cerebral blood flow response to increased synaptic activity, suggesting a local influence for nNOS-derived NO beyond the specific coordination of presynaptic and postsynaptic neurons¹³³.

In addition to the physiological effects of nNOS-derived NO observed in the CNS, a number of pathophysiological effects have also been identified in recent years. Most prominently, dysfunctional NO production by nNOS is heavily implicated in the pathological progression of cerebral ischemia. Disruption of PSD-95/nNOS coupling has been shown to be neuroprotective after MCAO in rodents, a finding consistent with the reduced cell death seen in nNOS^{-/-} mice after MCAO¹³⁴⁻¹³⁵. Similarly, in an MPP⁺ model of dopaminergic neurodegeneration, genetic deletion or pharmacological inhibition of nNOS proved effective in preserving neuronal viability¹³⁶⁻¹³⁷. Indeed, excessive nNOS activity is implicated in the abnormal regulation of a number of different proteins that contribute to neurodegeneration, including peroxiredoxin-2¹³⁸ (Prx2), X-linked inhibitor of apoptosis¹² (XIAP), glyceraldehyde-3-phosphate dehydrogenase⁷ (GAPDH), and parkin¹³⁹. Much of this disproportionate synthesis of NO may be due to the high levels of extracellular glutamate present after initial cellular degeneration in the CNS: the resultant stimulation of surrounding neuronal NMDA receptors could activate nNOS into producing supraphysiological levels of NO in response¹⁴⁰. Considered as a whole, the evidence indicates that the difference between physiological and

pathophysiological function in the CNS may be regulated, at least in part, through nNOS-derived $\cdot\text{NO}$.

1.5 Mechanisms of Nitric Oxide-Based Signaling

1.5.1 Activation of sGC/cGMP Cascades

Soluble guanylate cyclase (sGC) is one of two main subtypes of the guanylyl cyclase enzyme, which is responsible for catalyzing the production of cGMP and pyrophosphate from GTP. Three subunits of the enzyme have been identified: α_1 , α_2 , and β_1 . A functional complex of the enzyme consists of a heterodimer of α and β subunits, resulting in two isoforms of sGC: $\alpha_1\beta_1$ and $\alpha_2\beta_1$. Both isoforms are expressed in the brain, and exhibit regional differences in expression: $\alpha_1\beta_1$ is more prominent in the caudate/putamen and nucleus accumbens, while $\alpha_2\beta_1$ is expressed at higher levels in the hippocampus and olfactory bulb¹⁴¹⁻¹⁴².

Nitric oxide acts on sGC through direct binding to the N-terminal heme-containing domain of the enzyme's β_1 subunit. The heme group of sGC shows exquisite sensitivity for $\cdot\text{NO}$ over O_2 , with the ability to detect $\cdot\text{NO}$ in the presence of a 10,000-fold excess of O_2 and an EC_{50} for $\cdot\text{NO}$ between 1-2 nM¹⁴³⁻¹⁴⁴. One of the current models postulates that binding of $\cdot\text{NO}$ to the heme group leads to the release of an Fe^{2+} -ligated histidine residue from the heme group, leading to conformational changes in the enzyme that are transduced to the catalytic domain. Subsequently, this allows for the binding of other $\cdot\text{NO}$ molecules to

lower-affinity sites on the protein, and results in significantly upregulated cGMP production¹⁴⁵. Presynaptically, NO synthesis and subsequent sGC activity is primarily initiated by action potential-dependent Ca^{2+} flux through N-type channels¹⁴⁶. Postsynaptic sGC activity is chiefly dependent on NMDA receptor-mediated Ca^{2+} flux, due to its association through PSD-95 to both the NMDA receptor and nNOS¹⁴⁷.

NO -dependent changes in cGMP production are vital in mediating downstream biological processes in the CNS. For example, they can regulate Na^+ , Ca^{2+} , and K^+ flux through CNG and HCN channels, thereby modulating neuronal excitability¹⁴⁷⁻¹⁴⁸. Additionally, they can lead to autoregulation of cyclic nucleotide levels through the activation of specific phosphodiesterases (including PDE2, PDE5, and PDE6), leading to reductions in cAMP/cGMP signaling¹⁴⁹. Alternatively, NO can exert its effects on cGMP signaling cascades through the regulation of cGMP-dependent protein kinase (PKG) activity. This process has been implicated in a variety of different functions in the CNS, including vesicular GABA release¹¹², K^+ flux through Kv1.1/1.2 channels¹⁵⁰, ryanodine receptor (RyR)-dependent Ca^{2+} release from internal stores¹⁵¹, and other pathways¹⁵².

Despite the evidence supporting cGMP signaling as a relevant NO effector, it was soon recognized that it was not the *only* pathway available for NO -dependent regulation of biological function. Inhibition of cGMP synthesis did not completely block many of the NO -dependent effects observed in brain physiology, indicating a yet-undiscovered regulatory avenue by NO that

extended beyond phosphorylation-dependent signaling cascades¹⁵³⁻¹⁵⁶.

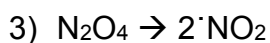
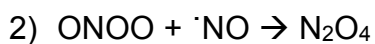
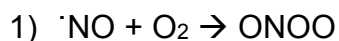
Eventually, this led to the identification of S-nitrosylation as another mechanism through which $\cdot\text{NO}$ could influence protein structure and function.

1.5.2 Protein S-Nitrosylation

S-nitrosothiols were first identified by Tasker and Jones in 1909, after treatment of ethane-thiol with nitrosyl chloride¹⁵⁷. The biological utility of this chemical species was not noted until a series of studies by Ignarro and colleagues in the 1980s, which demonstrated novel properties for S-nitrosothiols in smooth muscle relaxation and anti-platelet aggregation¹⁵⁸⁻¹⁵⁹. These effects were initially attributed to the activation of sGC by $\cdot\text{NO}$ released from the thiol. However, by 1990 Kowaluk and Fung concluded that a number of the bioactive effects of S-nitrosothiols could not be solely accounted for by decomposition to $\cdot\text{NO}$ ¹⁶⁰. These findings were soon extended by Stamler and colleagues in 1992, who found that protein thiols could undergo the transformation to S-nitrosothiols under physiological conditions and thereby extend the half-life of $\cdot\text{NO}$: the resultant S-nitrosoproteins also possessed vasodilatory and platelet aggregation effects¹, similar to EDRF/ $\cdot\text{NO}$. Subsequent identification of endogenous S-nitrosoalbumin in blood plasma confirmed the *in vivo* biosynthesis of S-nitrosoproteins, and opened up a new field of $\cdot\text{NO}$ biology for further study¹⁶¹.

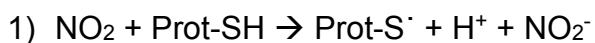
1.5.2.1 Mechanisms of Protein S-Nitrosylation and De-Nitrosylation

Several biochemical mechanisms of protein S-nitrosocysteine formation (Prot-SNO) have been proposed, both nonenzymatic and enzymatic. The first involves the autooxidation of $\cdot\text{NO}$ through its reaction with O_2 , resulting in the generation of a series of nitrogen oxides¹⁶².



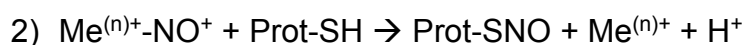
N_2O_3 is considered a potent nitrosating agent, and is thought to react with protein thiols (Prot-SH) to generate protein S-nitrosocysteine. The rate constant for N_2O_3 formation in aqueous environments ($2\text{-}5 \times 10^6 \text{ M}^{-2} \text{ s}^{-1}$) is thought to render it impractical under physiological conditions, due to the rate-limiting formation of NO_2 ^{17, 163-166}. Despite this limitation, it may be favored in localized cellular compartments, due to a few key factors. The reaction rate for N_2O_3 generation is very sensitive to reactant concentration, especially that of $\cdot\text{NO}$. Additionally, the rate constant for the reaction can increase up to 300-fold in hydrophobic environments such as biological membranes¹⁶⁷⁻¹⁶⁹. Given the lipophilic nature of $\cdot\text{NO}$, both of these requirements may be met at sufficient enough levels in compartmentalized membrane environments to generate local nitrosating

species¹⁷⁰. Alternatively, another mechanism for S-nitrosylation relies on the generation of protein thiyl radical (Prot-S[•]) from the reaction between NO₂ and a cysteine thiol¹⁷¹.



However, this process still relies on the rate-limiting formation of NO₂, leaving it with the same restrictions as N₂O₃.

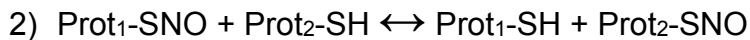
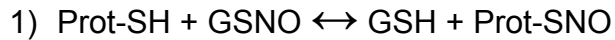
A second demonstrated mechanism for protein S-nitrosylation depends on the direct reaction of [•]NO with a transition metal (Me⁽ⁿ⁺¹⁾⁺), such as Fe³⁺ or Cu²⁺. This results in the reduction of the metal, as well as the generation of a nitrosonium (NO⁺) ion, at rates more physiologically relevant than those observed for NO₂/N₂O₃ formation. The nitrosonium, subsequently, is free to react with local cysteine thiols¹⁷²⁻¹⁷⁴.



Another related mechanism for protein S-nitrosylation relies on the generation of dinitrosyl-iron complexes from [•]NO and free iron pools, which can then lead to subsequent protein S-nitrosylation¹⁷⁵⁻¹⁷⁷.

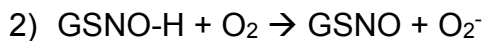
A third mechanism of protein S-nitrosylation involves the reversible transfer of a nitrosonium group between cysteine thiols, referred to as trans-

nitrosylation. The source of the nitrosonium can be either a low molecular weight S-nitrosothiol, such as S-nitrosoglutathione (GSNO), or another protein S-nitrosocysteine (the second scenario is referred to as protein-assisted trans-nitrosylation: this includes protein de-nitrosylation as well).



Intracellular pools of glutathione (GSH) and GSNO are major endogenous sources of nitrosonium acceptors and donors, respectively^{161, 178-181}. Together with existing protein thiols and S-nitrosothiols, they provide the substrates necessary for the regulation of cellular processes.

The biosynthetic mechanisms underlying intracellular GSNO formation under aerobic conditions remain unclear, although it may require either $\cdot\text{NO}$ autooxidation to N_2O_3 or the reaction of $\cdot\text{NO}$ with a glutathionyl radical ($\text{GS}\cdot$). Alternatively, it may be generated through the generation of a thionitroxide intermediate¹⁸² (GSNO-H), which can then reduce molecular oxygen and result in superoxide (O_2^-) and GSNO.



GSNO levels are maintained by several different enzymes, such as S-nitrosoglutathione reductase (GSNO-R), carbonyl reductase 1 (CR1), thioredoxin

(Trx), and γ -glutamyl transpeptidase (γ -GT). In doing so, they may indirectly influence protein S-nitrosylation in a number of different tissues through the regulation of available nitrosonium from GSNO¹⁸³⁻¹⁸⁶.

Protein-assisted trans-nitrosylation represents an additional avenue of NO-dependent signal transduction, though one that remains relatively unexplored. One of the first trans-nitrosylation-dependent “cascades” described involves the regulation of caspase-dependent cell death by thioredoxin. Thioredoxin modulates the Fas-induced activation of caspase-3 through trans-nitrosylation/de-nitrosylation of caspase-3, with caspase de-nitrosylation a prerequisite for apoptosis^{185, 187}. Similarly, GAPDH-mediated trans-nitrosylation of Siah has been identified as a key contributor to neuronal apoptosis^{7, 188}. A third recently elucidated pathway involves the assembly of proteins in the interferon-gamma (IFN- γ)-activated inhibitor of translation (GAIT) complex, a process dependent on S100A9-mediated trans-nitrosylation¹⁸⁹. Additional studies have identified candidate de-nitrosylases in the regulation of cellular S-nitrosothiol levels, including protein disulfide isomerase, xanthine oxidase, superoxide dismutase, and glutathione peroxidase¹⁹⁰⁻¹⁹³. Given the large number of S-nitrosoproteins already identified, these pathways may only represent a small fraction of the trans-nitrosylation-dependent processes involved in physiological function.

1.5.2.2 Selectivity of S-Nitrosylation

Although significant progress has been made in identifying the protein substrates of S-nitrosylation, the selectivity behind the endogenous modification of specific cysteine residues is not as well-described. Several attempts have been made to decipher the biophysical and biochemical requirements behind *in vivo* S-nitrosylation of cysteine residues with mixed results. Analysis of protein S-nitrosocysteines identified in the WT mouse liver found them to be overrepresented in α -helical structures, with locations in larger surface-accessible areas and proximal (within a 6-Å radius) to charged amino acids¹⁹⁴. However, these structural characteristics could not account for all S-nitrosocysteines identified, a finding confirmed in a later survey of S-nitrosocysteines identified in multiple mouse organs⁴⁻⁵. Additional studies have indicated that charged residues distal to specific cysteine residues could participate in protein-protein interactions that may facilitate S-nitrosylation of those cysteine residues, with proximal acid/base motifs thought to play a role in this process as well¹⁹⁵⁻¹⁹⁶. S-nitrosocysteines have also been found near metal centers of proteins^{176, 197-198}, a fact that may be important given the known ability of certain transition metals to coordinate to $\cdot\text{NO}$. Recently, a I/L-X-C-X₂-D/E motif for protein trans-nitrosylation has been discovered in members of the GAIT complex¹⁸⁹, which become S-nitrosylated by iNOS-derived $\cdot\text{NO}$ in response to IFN- γ and oxidized low-density lipoprotein (LDL_{ox}). When considered as a whole, the evidence suggests that multiple mechanisms may be involved in the

selectivity of S-nitrosocysteine formation in proteins. This, in turn, would result in the diverse subset of S-nitrosoproteins detected thus far.

1.5.2.3 S-Nitrosylation and Protein Function in the CNS

As an alternative pathway for NO -dependent cell signaling, S-nitrosylation has garnered intense interest in the CNS. A survey of prior reports has yielded more than 170 proteins with 323 S-nitrosocysteine residues *in vivo* in rodent brain. Of these proteins and sites, only 120 proteins with 181 sites were found under physiological conditions, suggesting a biased association in the literature between S-nitrosylation and cell death in the brain. On the other hand, additional roles have also been observed for protein S-nitrosylation in neuronal development and synaptic transmission. Collectively, the existing information suggests a functional pleiotropy for this modification during both normal physiology and disease in the CNS, similar to phosphorylation or acetylation¹⁹⁹⁻²⁰⁰.

Several groups have focused on the contributions of S-nitrosylation of specific protein targets to neurodegeneration. These include, among others, the inhibition of parkin E3 ubiquitin ligase activity¹³⁹, GAPDH-induced apoptosis^{7, 201}, caspase activation via XIAP inhibition¹², PTEN-dependent regulation of PI3/Akt signaling²⁰², and MEF2-mediated transcriptional regulation¹⁰. The widespread effects of S-nitrosylation in multiple processes, ranging from transcriptional repression of specific genes to perturbations in numerous cell survival pathways, imply a large contribution from NO to the pathological effects observed in many

chronic neurological disorders (such as Alzheimer's or Parkinson's disease). Despite such findings, however, it is the effects of endogenous S-nitrosylation on physiological function that are gaining recognition for their importance in refining and maintaining critical biological processes within the brain.

In recent years, the significance of this modification in normal CNS development has become increasingly apparent. S-nitrosylation of transcription factor MEF2A prevents it from binding to specific promoters, leading to decreased expression of the nuclear receptor *tailless* and subsequent impairments in neurogenesis and neuronal differentiation¹⁰. Transduction of the BDNF/CREB-dependent signaling cascade is achieved through the S-nitrosylation of effector molecules, including sirtuin-1 (SIRT1) and histone deacetylase 2 (HDAC2), resulting in gene expression changes that affect neuronal differentiation and axodendritic morphology^{201, 203-204}. Furthermore, axonal retraction is partly guided via S-nitrosylation of the light chain of microtubule-associated protein 1B (MAP1B), thereby affecting growth cone dynamics during CNS maturation²⁰⁵. Given this evidence, a case can be made for protein S-nitrosylation as a “synaptic tag”, linking neuronal signaling (via glutamate and postsynaptic NO production by nNOS) to longstanding changes in synaptic plasticity²⁰⁶.

Perhaps most interesting are the documented effects of S-nitrosylation on protein function at the synapse itself. It regulates cation flux through NMDA²⁰⁷ and AMPA²⁰⁸ receptors, as well as the attachment of scaffold protein PSD-95 to

the postsynaptic plasma membrane¹¹. Additionally, it governs postsynaptic AMPA receptor trafficking²⁰⁹⁻²¹⁰ and gephyrin clustering²¹¹ in response to glutamatergic and GABAergic activity, respectively. Finally, D-serine production is decreased by S-nitrosylation of serine racemase²¹², leading to its reduced availability as a coagonist for synaptic NMDA receptor activation²¹³. Taken together, these data suggest S-nitrosylation as a potent coordinator of both short-term and long-term responses to synaptic transmission. However, since previous reports of the modification in the CNS primarily focused on analyses of single proteins, investigation of this hypothesis may be better aided by large-scale *in vivo* proteomic identification of endogenously S-nitrosylated proteins from the brain. Most of the approaches developed thus far for such studies are outlined in the next chapter. Moreover, it leads to the central questions of our study in Chapter 3, namely:

- 1) Which targets of S-nitrosylation in the brain are involved in the regulation of synaptic function?
- 2) What is the source of $\cdot\text{NO}$ utilized for the modification of these proteins (this may also contribute to spatial localization/modification of these proteins)?
- 3) What are the functional effects of this modification in these proteins, and how do they contribute to neurotransmission as a whole?

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CHAPTER 2

STRATEGIES AND TOOLS TO EXPLORE PROTEIN S-NITROSYLATION

By

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Abstract

A biochemical pathway by which nitric oxide accomplishes functional diversity is the specific modification of protein cysteine residues to form S-nitrosocysteine. This post-translational modification, S-nitrosylation, impacts protein function and location. Despite considerable advances with individual proteins, the biological chemistry and the structural elements that govern the modification of specific cysteine residues *in vivo* are vastly unknown. Moreover comprehensive studies exploring protein signaling pathways or interrelated protein clusters that are regulated by S-nitrosylation have not been performed on a global scale. To provide insights to these important biological questions, sensitive, validated and quantitative proteomic approaches are required. This review summarizes current approaches for the global identification of S-nitrosylated proteins.

Key Words

S-nitrosylation

Proteomics

Mass spectrometry

Cysteine post translational modification

Nitric oxide

Highlights

- S-nitrosylation of cysteine residues an important regulator of protein function
- Description of methods to identify S-nitrosylated proteins
- Mass spectrometry-based proteomics essential for further studies of S-nitrosylation biology

2.1 Introduction

Since its discovery nitric oxide has become increasingly evident as a major regulator of physiological function. The effects of nitric oxide on physiology are exerted primarily through two molecular mechanisms, comprised of cyclic GMP (cGMP)-dependent signaling cascades and post-translational modification (PTM) of proteins. Initially, nitrosylation of the heme iron in soluble guanylate cyclase was found to activate the enzyme to generate cGMP, thereby regulating the function of a number of cGMP-dependent signaling pathways. However, in recent years the importance of nitric oxide-derived post-translational modifications of proteins has gained recognition as mediators of protein function.

One of these modifications, S-nitrosylation, is defined as the covalent addition of a nitric oxide equivalent to the thiol side chain of cysteine [1]. This modification has been shown to alter protein activity, protein-protein interactions, and sub-cellular localization under physiological and pathological conditions [2, 3, 4, 5]. Additionally, evidence indicates that S-nitrosylation is reversible and is regulated in a temporal and spatial sense, reminiscent of other post-translational modifications such as phosphorylation [6, 7, 8]. Despite these indications of the emerging significance of S-nitrosylation, little is known regarding the proximal mechanisms of in vivo formation as well as how selectivity, in terms of directing modification to specific cysteine residues, is achieved. To improve our understanding of the formation and selectivity of this post-translational modification in vivo, global interrogation of S-nitrosoproteomes can be exceptionally valuable. Below we review the methodologies for the global identification of S-nitrosylated proteins (Table 2.1), and discuss potential utilities of proteomic-derived data.

2.2 The Biotin Switch and Its Variations

2.2.1 The Biotin Switch

The biotin switch [9] was the first and is the most commonly used technique for identifying S-nitrosylated proteins. Three steps constitute the basic principles of the method: 1) initially, proteins in a purified preparation or complex mixture are denatured by SDS (or urea, which is more amenable to downstream mass spectrometry analysis, 10); to expose protein cysteine residues. Subsequently, the reduced thiols in these proteins are blocked by reaction with reagents such as methyl methanethiosulfonate (MMTS), N-ethylmaleimide (NEM), or iodoacetic acid (IAA). 2) Following this blocking step, S-nitrosylated -cysteine residues are selectively reduced by treatment with ascorbate. 3) The ascorbate-reduced cysteine residues are then reacted with biotin-HPDP (N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)-propionamide). The conjugation of these protein cysteine residues to biotin permits enrichment of the modified targets from a complex mixture using avidin-based affinity capture. The affinity enriched preparations can be probed with antibodies, if the protein targets of interest are known, or digested with trypsin and subjected to liquid chromatography tandem mass spectrometry (LC-MS/MS). One added benefit of this method is that it is amenable to stable isotope labeling with amino acids in cell culture (SILAC) in order to quantitatively determine the levels of S-nitrosylation for specific cysteine residues via mass spectrometry-based approaches [11].

2.2.2 Biotin Switch-based Peptide Identification

The original biotin-switch method did not provide the means to identify the site(s) of modification in S-nitrosylated proteins. Identification of these sites is

the ultimate qualifier for the unambiguous assignment of S-nitrosylated proteins. Site-specific identification enables mutational analysis to explore the functional role of this modification. Two similar approaches to achieve site-specific identification of modified cysteine residues have been developed based on the biotin switch [12, 13]. For both methods, proteins are first digested with trypsin and the resultant peptides are incubated with avidin or its derivatives. These peptides are then eluted and subjected to LC-MS/MS to determine the site of modification.

2.2.3 The His-Tag Switch

Since the original description of the biotin switch methods, several variations have been developed. One of these variants, the His-Tag switch, [14] begins with the blocking of free thiols by NEM, followed by ascorbate reduction of S-nitrosylated cysteine residues. It diverges from the biotin switch, however, by treating these ascorbate-reduced cysteines with a conjugate of iodoacetate and a His-containing peptide. Proteins containing this “His-tag” are then enriched through affinity chromatography in a nickel column, after which they are eluted and subjected to 1-dimensional (1D) gel electrophoresis. In-gel digestion with trypsin is then performed. Trypsin, in addition to cleaving the peptide backbone also facilitates cleavage of part of the alkylating agent, resulting in a mass increase of the cysteine residue by 271.12 Da, which is used to identify the modified peptide by LC-MS/MS. This method thus provides another approach for identifying the site(s) of modification in S-nitrosylated proteins.

2.2.4 Fluorescence-based Detection (DyLight, Cyanine, and AMCA-based Methods)

Another method based on the biotin switch relies on 2-dimensional differential gel electrophoresis (2D-DIGE, 15). The initial steps of this approach are identical to the biotin switch. After blocking by MMTS (or NEM) S-nitrosylated-cysteine residues are reduced by ascorbate. However, instead of biotin-HPDP, a set of DyLight maleimide sulfhydryl reactive fluorescent compounds are used to react with the newly reduced cysteine residues. Individual samples labeled with a DyLight fluorescent compound are separated on a 2D gel. By comparing the fluorescent intensity of a single spot in the gel, a relative assessment of protein S-nitrosylation levels can be made. Another variation of this method employs fluorescent cyanine maleimide sulfhydryl reactive compounds instead of the DyLight compounds [16].

An additional iteration of this “fluorescence switch” utilizes 7-amino-4-methyl coumarin-3-acetic-acid (AMCA)-HPDP to label S-nitrosylated cysteine residues after ascorbate reduction [17]. In this method, samples are resolved on either 1D or 2D gels after labeling. Subsequently, the gel is subjected to UV illumination, which activates the AMCA fluorophore and allows it to be directly visualized. All three versions of the “fluorescence switch” method allow for relative quantification of S-nitrosylation levels of proteins between samples through comparison of fluorescent intensities. However, in each case the site(s) of S-nitrosylation in specific proteins remains elusive, thereby rendering further studies of the functional impact of this modification challenging.

2.2.5 d-Switch

In addition to the capabilities offered by a combination of SILAC labeling and the biotin switch, another quantitative method was developed and tested in a purified protein preparation [18]. In this technique, reduced cysteine residues in recombinant glutathione S-transferase P1 (GST-P1) are labeled with NEM. S-nitrosylated cysteine residues in GST-P1 are then subjected to ascorbate reduction, followed by treatment with deuterated (d₅)-NEM and in-gel trypsin digestion. Mass spectrometry is then used to determine the relative amounts of peptides containing only d₅-NEM versus those containing both d₅-NEM and NEM, providing a quantitative assessment of S-nitrosylation.

2.2.6 SNO-RAC

Recently, another approach was developed by Stamler and colleagues to enrich for S-nitrosylated proteins from a complex mixture [19]. Referred to as “SNO-RAC,” this method relies on the conjugation of reduced cysteine residues to a solid support, such as thiopropyl sepharose. The first two steps of the protocol are identical to the biotin switch. However, the ascorbate-reduced cysteine residues are incubated with thiol-reactive resins, resulting in a covalent disulfide linkage. At this point, the proteins can be eluted and analyzed by western blotting. Alternatively, the disulfide linkage allows for on-resin trypsin digestion of bound proteins, resulting in site-specific identification of modified cysteine residues by LC-MS/MS. Compared to the traditional biotin switch, SNO-RAC has a better sensitivity for proteins with higher mass (>100 KDa). When combined with iTRAQ labeling, it can also report on the S-nitrosylation/de-nitrosylation of specific cysteine residues on a global scale [20].

2.2.7 Overview of the Biotin Switch-based Methods

Application of the biotin switch method and its various iterations to the identification of endogenously S-nitrosylated proteins (defined here as proteins in cell lysate or tissue homogenate without nitric oxide or trans-nitrosylating donor treatment) has yielded 135 targets from multiple organs and cell types. Within this subset of proteins, 82 sites have been reported from 63 proteins, illustrating the importance of the biotin switch in exploring the S-nitrosoproteome. However, three potential issues have been discussed with regard to these methods. First, the efficiency/sensitivity of this assay relies on complete blocking of reduced cysteine residues. Incomplete blocking will result in false identification, which can be minimized by the inclusion of negative controls such as pretreatment with ultraviolet (UV) photolysis or dithiothreitol (DTT). Second, the efficiency of ascorbate reduction has been questioned [21, 22, 23], suggesting decreased sensitivity of the method. The concerns over ascorbate reduction are compounded by its potential ability to reduce disulfides [24, 25], leading to false identification. Again, the inclusion of negative controls (listed above) as well as samples not treated with ascorbate can be employed. Alternatively, sinapinic acid has been used to treat cell lysates in place of ascorbate as a more selective method of reducing S-nitrosylated cysteine residues [26]. Third, there is the possibility of disulfide exchange after ascorbate reduction, leading to false identification of modified cysteine residues [20]. The use of methods that rely on a direct reaction with S-nitrosylated residues without requiring ascorbate reduction may overcome this concern. Taken together, these potential methodological challenges stimulated the development of alternative approaches for exploring the S-nitrosoproteome.

2.3 Direct Detection of S-nitrosylation by Mass Spectrometry

Theoretically, many of the concerns noted with the biotin switch and other chemical derivatizations can be avoided by direct detection of S-nitrosocysteine by mass spectrometry. In practice, such an approach remains challenging and has been possible primarily for isolated proteins. Certain MS approaches such as matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS, where the energy needed for peptide ionization also causes the loss of nitric oxide from the cysteine residues, can be employed. The reduction in mass by 29 Da in peptides after ionization is then diagnostic of S-nitrosylated sites. Within the last five years, a few groups [27, 28] have been able to directly identify S-nitrosylated cysteine residues by electrospray ionization quadrupole time-of-flight (ESI-QTOF) MS. However, such investigations were limited to synthetic proteins and/or peptides, or were only able to identify a few sites of S-nitrosylation from a complex mixture, rendering them unsuitable in their current form to proteome-wide identification of S-nitrosylated protein targets.

2.4 Gold Nanoparticle-based Enrichment

A method that relies on a direct reaction of S-nitrosocysteine residues with gold nanoparticles (AuNPs) has been developed for identifying sites of S-nitrosylation in purified protein preparations [29]. In this method, reduced cysteine residues are initially alkylated by iodoacetamide (IAM), after which the protein is subjected to proteolysis. Following this digestion, peptides are incubated with AuNPs to selectively react with the S-nitrosylated cysteine residues, generating free nitric oxide and peptides conjugated to the AuNPs. The nanoparticles are then treated with DTT to elute bound peptides, which are

subsequently analyzed by mass spectrometry to identify sites of modification. Despite the potential advantages offered by this method, two drawbacks exist. First, the AuNPs react with both S-nitrosylated and S-glutathionylated cysteines, providing a challenge for absolute assignment of specific post-translational modifications to these residues. Second, this approach has not yet been applied to complex mixtures. Nonetheless, it holds excellent promise for site-specific identification of modified cysteine residues in S-nitrosylated proteins.

2.5 Phosphine-based Direct Labeling of S-Nitrosylated Proteins

To overcome the reliance of previous methods on the complete blocking of reduced cysteine residues (either by MMTS, NEM, or other reagents), Zhang and colleagues [30] introduced another approach to directly label S-nitrosylated cysteine residues in cell extracts. For this particular method, S-nitrosylated cysteine residues are reductively ligated (in the presence of water) with a biotin-labeled phosphine substrate, resulting in the generation of a sulfenamide product and thiolate. The sulfenamide and thiolate then spontaneously react to provide a stable disulfide (conjugated to biotin) at the formerly S-nitrosylated cysteine residue. Avidin-based enrichment of these labeled proteins is performed, and captured proteins are then resolved on a 1D gel. This particular approach is advantageous in its reactive specificity toward S-nitrosylated cysteine residues in the absence of blocking. Following the introduction of this method, a number of phosphine-based compounds have been developed that directly react with S-nitrosylated -cysteine residues [31, 32, 33]. Although these approaches have yet to identify endogenously modified sites, these compounds offer intriguing possibilities to explore protein S-nitrosylation *in vivo*.

2.6 Organomercury-Based Capture

After considering some of the limitations inherent in the biotin switch technique and its successors, we introduced novel complementary methods for capturing S-nitrosylated proteins and identifying their sites of modification [34]. In this protocol, phenylmercury compounds (either conjugated to an agarose solid support or to polyethylene glycol-biotin) react directly with S-nitrosocysteine residues to form a stable thiol-mercury bond [35]. The first step of this procedure is the same as that of the biotin switch. Following the blocking step, proteins are incubated with either the organomercury-conjugated resin (MRC) or a soluble phenylmercury-polyethyleneglycol-biotin (mPEGb) compound. After formation of the thiol-mercury bond, a number of options are available. S-nitrosylated proteins can be enriched by either the MRC approach or by avidin-based affinity capture (for mPEGb), and eluted using beta-mercaptoethanol to reduce the thiol-mercury bond. Eluted proteins are then subjected to 1D gel electrophoresis, in-gel trypsin digestion, and LC-MS/MS analysis. In order to identify the specific cysteine residues modified by S-nitrosylation, a slightly altered protocol is applied. After incubation of blocked proteins with the phenylmercury-based reagents, proteins are then digested with trypsin on-resin or in-solution. The resultant peptides are either eluted with mild performic acid to oxidize the cysteine in the thiol-mercury bond to cysteic acid (for the MRC approach), or subjected to a combination of avidin-based affinity capture and performic acid oxidation. In both cases, the cysteic acid generated in captured peptides is used as the MS signature to identify the cysteine modified by S-nitrosylation. Application of these methods yielded 328 endogenous sites of S-nitrosylation in 192 proteins in the wild-type mouse liver [34]. The method relies on the inclusion

of negative controls and the reporting of the percentage of peptides identified as false positives (those shared between negative controls and experimental samples).

Advantages of this approach include the fact that it circumvents the ascorbate reduction step. Additionally, it provides an opportunity to pinpoint the modified cysteine residue using the MS/MS signature of the cysteic acid (C+48). Since both proteins and peptides are identified independently, the peptides can be matched to the proteins and thus not rely on a single peptide for protein identification, as in previous methods [12, 13].

2.7 Overview of mass-spectrometry-based methodologies for detection of S-nitrosylated proteins

As with any other PTM-based proteomic studies, there are some potential caveats associated with global analyses of *in vivo* S-nitrosylated proteins. (1) Up to this point, only a few methods afford sensitivity for *in vivo* detection, and often investigators rely on the induction of S-nitrosylation by applying exogenous nitric oxide donors or trans-nitrosylating agents to cells and protein preparations. Unfortunately, such studies provide only putative sites of modification, not necessarily those modified *in vivo* [36]. (2) Some methods, but not all, do not provide the site of modification. The identification of the site not only provides confidence for the correct identification of the protein but also enables follow-up studies, such as mutational analysis, to explore biological function. (3) Negative controls and the reporting of false identification rate (FIR) must be routinely evaluated. (4) We must also consider the possibility that even a method which identifies the sites of modification, with appropriate negative controls and low

FIR, may still only report a subset of the modified proteins that are either most abundant or more stable. Despite these limitations, it is still worthwhile to pursue inquiries into the S-nitrosoproteome, if only to provide more information about the structural and functional importance of this modification *in vivo*.

2.8 Conclusions

Compared to widespread studies of PTMs such as protein phosphorylation, glycosylation, or even acetylation, the field of S-nitrosylation represents relatively uncharted territory. Within the last few years, however, this area of research has become quite prominent due to improvements in the detection of S-nitrosylated cysteine residues. By applying mass spectrometry-based proteomics, a greater appreciation of the biological significance of this modification is emerging. Additional work is needed to improve the sensitivity of these methods. Armed with sensitive and specific techniques, the S-nitrosoproteome of multiple organs and/or cell types can be investigated. The rich data from such studies can be analyzed in a variety of ways. Structurally, it can reveal significant new insights on elements that guide the selectivity of the modification. Additionally, it can also provide clues to potential biochemical reactions that derive the modification as well as explore the relative stability of different sites of modification. Functional analyses can uncover pathways and functional clusters of S-nitrosylated proteins, as well as their cellular location(s) [37], allowing for development of novel hypotheses that can be tested using more targeted approaches.

Table 2.1

Method	Principles	Comments
1) Biotin Switch Technique (BST): The original method and several variations thereafter: -Detergent-free BST -SNOSID -d-Switch -His-Tag Switch -Fluorescent Switch/2D-DIGE -SNO-RAC	1) Blockage of free thiols with thiosulfonate MMTS 2) Ascorbate reduction of S-nitrosocysteine. 3) Reaction of ascorbate-generated free cysteine with biotin-HPDP (or other thiol-reactive compounds). 4) Enrichment of labeled proteins or peptides. 2D-DIGE to assess relative changes in S-nitrosylation levels. 5) Western blot or LC-MS/MS to identify protein targets. 6) Peptide enrichment allows site-specific identification of modified cysteine residues.	Collectively 135 endogenously-modified protein targets have identified. 82 endogenous sites on 63 proteins were also identified Proteomics applicability. Reliance on selective reduction of SNO moiety prior to labeling, which has been discussed previously (20-26).
2) Direct Detection by ESI-QTOF Mass Spectrometry	Direct detection of SNO moiety on modified proteins.	Site-specific identification Limited to isolated proteins or peptides.
3) Phosphine-based derivatization	Specific reaction of SNO moiety on proteins with phosphine-based compounds without blocking free thiols.	Direct reaction of phosphine compounds with S-nitrosocysteine provides selective one-step labeling of modified residues. Potential applicability for mass-spectrometry-based proteomics needs to be explored.
4) Gold Nanoparticles (AuNPs)	1) Blockage of free thiols with IAM. 2) Enrichment of SNO-proteins via reaction of S-nitrosocysteine with AuNPs. 3) Trypsin digestion and elution of bound peptides. 4) LC-MS/MS to identify sites of modification.	Site-specific identification. AuNP reactivity toward S-nitrosocysteine and S-glutathionylated cysteine residues prevents absolute assignment of correct PTM. Applied only to purified protein preparations, not complex mixtures.
5) Organomercury-based Affinity Enrichment	1) Blockage of free thiols with MMTS. 2) Reaction with organomercury reagents. 3) LC-MS/MS to identify independently protein targets and sites of modification.	Site-specific identification of 328 endogenous S-nitrosylated peptides in 192 proteins in a single experiment. Reliance on organomercury-SNO chemistry; requires negative controls.

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CHAPTER 3

REGULATION OF BRAIN GLUTAMATE METABOLISM BY NITRIC OXIDE AND S-NITROSYLATION

By

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3.1 Introduction

Nitric oxide (NO) is an important physiological regulator of biological function in multiple tissues. NO-mediated signaling contributes to synaptic transmission and cerebrovascular coupling in the CNS (1-4). The biological effects of NO are primarily achieved through two molecular mechanisms: (i) the activation of soluble guanylate cyclase and downstream cGMP-dependent signaling cascades and (ii) S-nitrosylation, the post-translational modification of reduced cysteine residues in proteins to generate S-nitrosocysteine. Protein S-nitrosylation has emerged as a vital mediator of protein function and signaling.

The neuronal isoform of nitric oxide synthase (nNOS) produces NO in the brain. Mice with a genetic deletion of nNOS (*nNOS*^{-/-}) demonstrate various phenotypic insufficiencies (5), including deficits in several forms of memory (6-9). Furthermore, these mice have decreased presynaptic/postsynaptic excitability (10-11) and synaptic plasticity (12-14), effects that may be due to impaired glutamatergic neurotransmission. Additionally, *nNOS*^{-/-} mice exhibit reduced neurodegeneration after cerebral ischemia, which may be because of reduced glutamate excitotoxicity (15-16). Therefore nNOS-derived NO could affect synaptic activity by regulating glutamate availability. Many of the effects of NO on neurotransmission occur independently of cGMP activation (17-19), suggesting another mechanism for NO-dependent regulation of neurotransmission.

Protein S-nitrosylation is an alternative biochemical and molecular pathway by which NO could influence neurotransmission in the CNS (20). Selective protein S-nitrosylation has been linked to a few postsynaptic processes including the regulation of NMDA receptor and AMPA receptor activity, synaptic

targeting of PSD-95, gephyrin clustering at GABAergic synapses, surface expression of AMPA receptors, and D-serine production (21-27). However, the biological functions of protein S-nitrosylation in the tripartite glutamatergic synapse remain mostly unknown. Here, we used mass spectrometry (MS)-based proteomic methodologies to identify protein S-nitrosocysteine residues in brain homogenates from wild-type, *nNOS*^{-/-} and *eNOS*^{-/-} mice. When coupled with metabolomic profiling, enzyme activity measurements, and site-directed mutagenesis, the proteomic data indicates that reversible protein S-nitrosylation regulates glutamate uptake, metabolism, conversion to glutamine, and glutamatergic transmission.

3.2 Results

Ontological analysis of brain S-nitrosylated proteins

Mass spectrometric analysis of endogenous S-nitrosylated proteins using organomercury-based enrichment approaches (28-30) unearthed 269 sites in 136 proteins in wild-type mouse brain, 135 sites in 95 proteins in *eNOS*^{-/-} brain, and 71 sites in 53 proteins in *nNOS*^{-/-} brain (Fig. 1A and Table 3.1). The reduction in the S-nitrosylation of residues and proteins in *nNOS*^{-/-} (74%) and *eNOS*^{-/-} mice (50%) implied that both NOS isoforms contributed substantially to endogenous protein S-nitrosylation (Fig. 2). To place these observations in biological context, we performed gene ontology analysis. First we constructed a reference mouse brain proteome consisting of 7,025 proteins curated from previously published reports (31-34) and our experimental data. Using this reference proteome as a background, we performed computational analysis using WebGestalt (35) and identified several molecular pathways and cellular

processes in which S-nitrosylated proteins were significantly enriched in the wild-type brain (Fig. 1B and 3). The computed ratio of enrichment for the top five pathways was similar between wild-type and *eNOS*^{-/-} brains but was reduced in *nNOS*^{-/-} brains (Fig. 1B). For example, the molecular processes associated with the regulation of neurotransmitter levels showed a 67% reduction in *nNOS*^{-/-} brains, a finding consistent with previous descriptions of synaptic deficits in the *nNOS*^{-/-} mice (6-14). Within the regulation of neurotransmitter level pathway we mapped S-nitrosocysteine residues in four proteins: excitatory amino acid transporter 2, glutamate dehydrogenase, mitochondrial aspartate aminotransferase, and glutamine synthetase (Fig. 1C). The first three proteins were S-nitrosylated in wild-type and *eNOS*^{-/-} mice, but not in *nNOS*^{-/-} mice, whereas glutamine synthetase showed reduced S-nitrosylation in *nNOS*^{-/-} mice as compared to wild-type and *eNOS*^{-/-} mice (Fig. 1D and Table 3.1). We confirmed the mass spectrometric-based identification of these S-nitrosylated proteins by Western blotting (Fig. 1D), and quantified the fraction of each protein modified by S-nitrosylation in wild-type brain (Fig. 1E). These four proteins are major contributors in the glutamate/glutamine cycle (36-39), the biological process responsible for managing the metabolic fate and availability of glutamate in the synapse. Overall, the proteomic and ontological analysis indicates that proteins involved in the glutamate/glutamine cycle are selectively S-nitrosylated by nNOS, implying a role for nitric oxide in the regulation of glutamate metabolism.

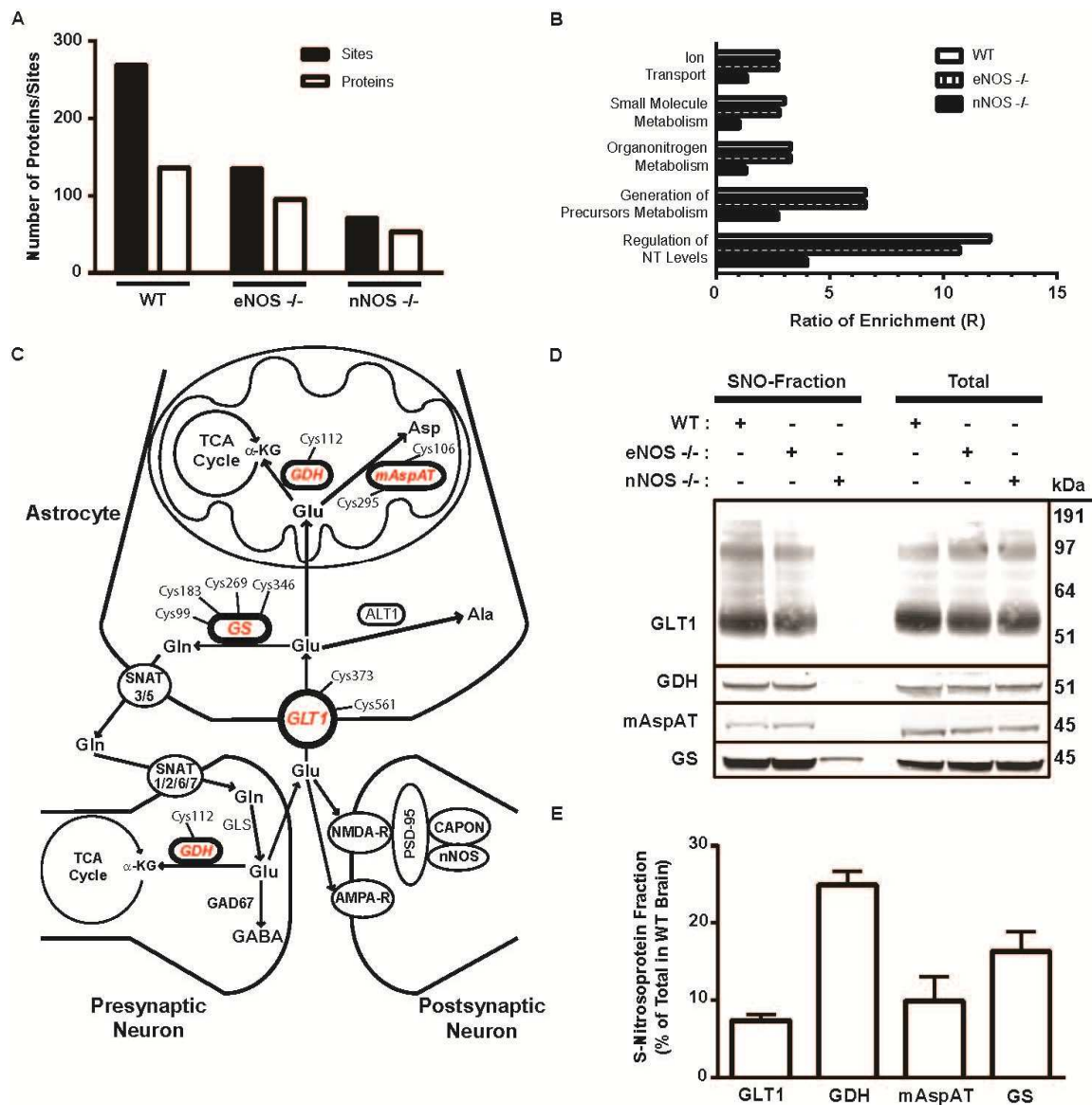


Figure 3.1. Proteomic Identification of Protein S-Nitrosocysteine residues and Gene Ontology Analysis of Modified Proteins in wild-type, eNOS^{-/-}, and nNOS^{-/-} Mouse Brain. **A)** Identification of S-nitrosylated proteins and their corresponding sites of modification in mouse brain (N = 6 mice per genotype). **B)** Gene ontology-based functional clustering of S-nitrosylated proteins in each genotype. The Ratio of Enrichment (R) score is calculated as the ratio of the observed number (O) of S-nitrosylated proteins with a specific gene ontology (GO) annotation in the WebGestalt database to the number of annotated proteins expected (E) to be in the S-nitrosocysteine proteome. Only the top five pathways in the wild-type mouse with the lowest p-values were considered (p < 0.01). NT = Neurotransmitter. **C)** Schematic of glutamate metabolism. Proteins are denoted by their short protein names, as assigned by Uniprot: GLT1 (excitatory amino acid transporter 2), GDH (glutamate dehydrogenase), mAspAT (mitochondrial aspartate aminotransferase), and GS (glutamine synthetase). S-nitrosylated proteins are italicized, with the associated sites of modification indicated. Gln = Glutamine, Glu = Glutamate, Asp = Aspartate, Ala = Alanine, α-KG = α-ketoglutarate, Cys = Cysteine. **D)** NOS-based dependence of S-nitrosylation of glutamate/glutamine cycle effectors. Modified proteins (denoted as the “SNO-Fraction”) within the aforementioned pathway were confirmed by Western blot against specific targets in all genotypes. The total abundance of the indicated protein targets in 30 μg of total homogenate did not obviously differ between genotypes. (N = 2 mice per genotype). **E)** Quantification of the relative S-nitrosylated fraction for each glutamate/glutamine cycle protein in wild-type mouse brain. Bars represent mean ± SEM (N = 3 mice).

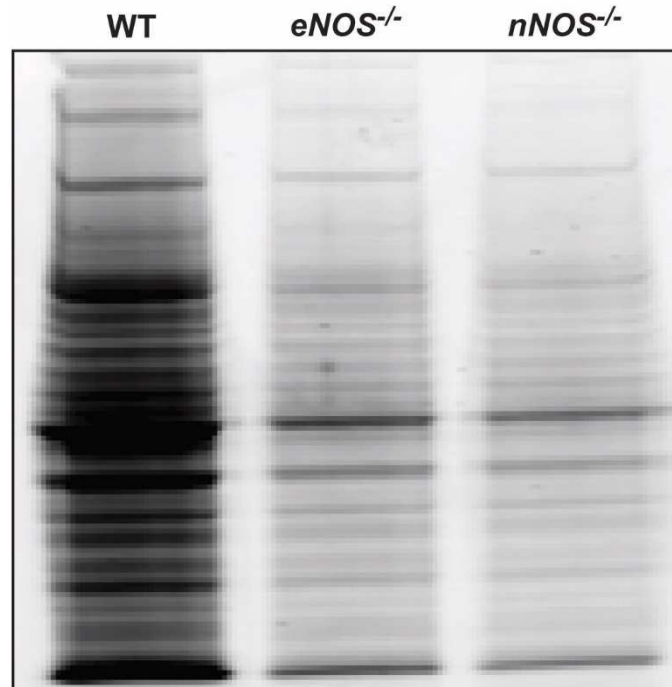


Figure 3.2. Reduction in the enrichment of S-nitrosylated proteins in *eNOS*^{-/-} and *nNOS*^{-/-} mice. Representative colloidal blue-stained gel showing the enrichment for S-nitrosylated proteins in forebrains from wild-type (WT), *eNOS*^{-/-}, and *nNOS*^{-/-} mice. Proteins were resolved by SDS-PAGE over a 4-cm distance on the gel, visualized by colloidal blue staining and scanned using the Odyssey Infrared Imaging System. The reduced intensity of protein staining in the lanes corresponding to *eNOS*^{-/-} and *nNOS*^{-/-} brain homogenates relative to wild-type homogenate, reflects the lower number of S-nitrosylated proteins in the brain of knockouts as compared to wild-type mice. This experiment was repeated twice with similar results (N=2 mice per genotype).

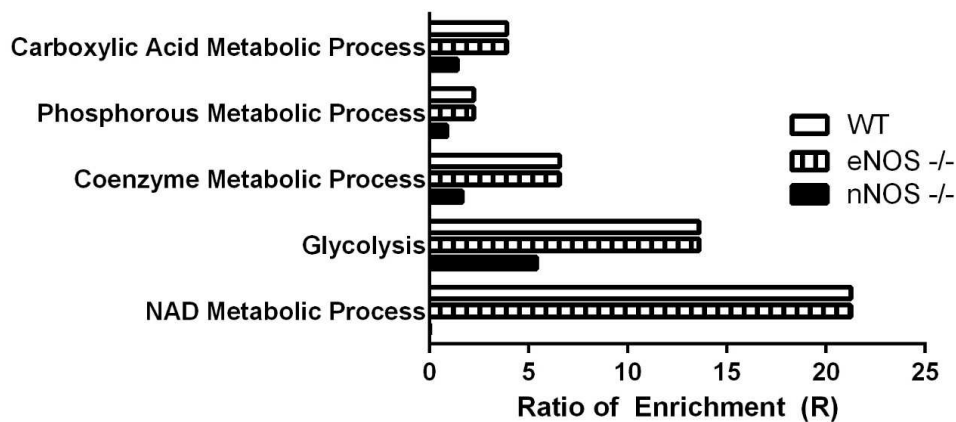


Figure 3.3. Additional biological processes and cellular functions identified for S-nitrosylated proteins in the mouse brain. Pathways identified by gene ontology analysis are involved in metabolic functions, including NAD/NADH metabolism, glucose metabolism and ATP synthesis/consumption. All pathways described were among the top ten pathways enriched in S-nitrosylated proteins ($p < 0.01$).

Regulation of Glutamate/Glutamine Metabolism by Nitric Oxide

To study the effects of nNOS-derived NO and protein S-nitrosylation on the glutamate/glutamine cycle we quantified the fractional isotopic enrichment of glutamate-associated metabolites in acutely-isolated hippocampal slices following treatment with [2-¹⁵N] L-glutamine (Fig. 4). Steady-state amounts of labeled glutamate-associated metabolites were quantified by HPLC and GS-MS approaches (40-41) under stimulatory (Mg²⁺-free) conditions in wild-type, *eNOS*^{-/-}, and *nNOS*^{-/-} mice. The three genotypes showed similar total amounts of glutamate, glutamine, aspartate, alanine and GABA (Fig. 5A), indicating a lack of gross metabolic dysfunction. However, the ratio of intracellular glutamine to glutamate was 30% higher in *nNOS*^{-/-} mice, reflecting potential perturbations in glutamate handling (Fig. 5B). ¹⁵N-fractional isotopic enrichment for alanine, aspartate, and glutamate ranging between 20-30% was significantly decreased in *nNOS*^{-/-} brain slices relative to the other two genotypes, confirming altered glutamate allocation in *nNOS*^{-/-} mice (Fig. 5C).

To further explore the influence of nNOS-derived NO on the glutamate/glutamine cycle, we performed enzymatic assays for glutamate dehydrogenase, mitochondrial aspartate aminotransferase and glutamine synthetase in mitochondrial extracts and brain homogenates (Fig. 5D). The activities of glutamate dehydrogenase and mitochondrial aspartate aminotransferase were significantly increased in *nNOS*^{-/-} mitochondrial extracts as compared to wild-type and *eNOS*^{-/-} mice (Fig. 5D). Trans-illumination of wild-type mitochondrial extracts with ultraviolet (UV) light, a treatment that effectively eliminates NO from S-nitrosocysteine residues (42-43), increased the enzymatic activities of glutamate dehydrogenase and mitochondrial aspartate

aminotransferase in wild-type extracts similar to that measured in *nNOS*^{-/-} extracts (Fig. 5D). We used selective inhibitors GTP (for glutamate dehydrogenase) and aminooxyacetic acid (for mitochondrial aspartate aminotransferase) to validate the specificity of the assays. Each inhibitor resulted in >95% loss of activity (Fig. 5D). Relative to wild-type homogenate, glutamine synthetase activity was decreased slightly in *nNOS*^{-/-} but not in *eNOS*^{-/-} brain homogenate (Fig. 5D). UV trans-illumination decreased the activity of GS in wild-type homogenate (Fig. 5D). The difference in the reduction of GS activity between *nNOS*^{-/-} mice and wild-type mice exposed to UV light most likely reflects contributions from additional nitric oxide synthase isoforms to the S-nitrosylation of GS *in vivo* (Fig. 1D and Table 3.1). Exposing wild-type homogenate to the glutamine synthetase inhibitor L-methionine sulfoximine (MSO) (44) resulted in a >95% loss of activity (Fig. 5D). Together, metabolomic profiling and enzymatic activity assays imply that selective nNOS-dependent S-nitrosylation of key proteins in the glutamate/glutamine cycle regulates glutamate metabolism.

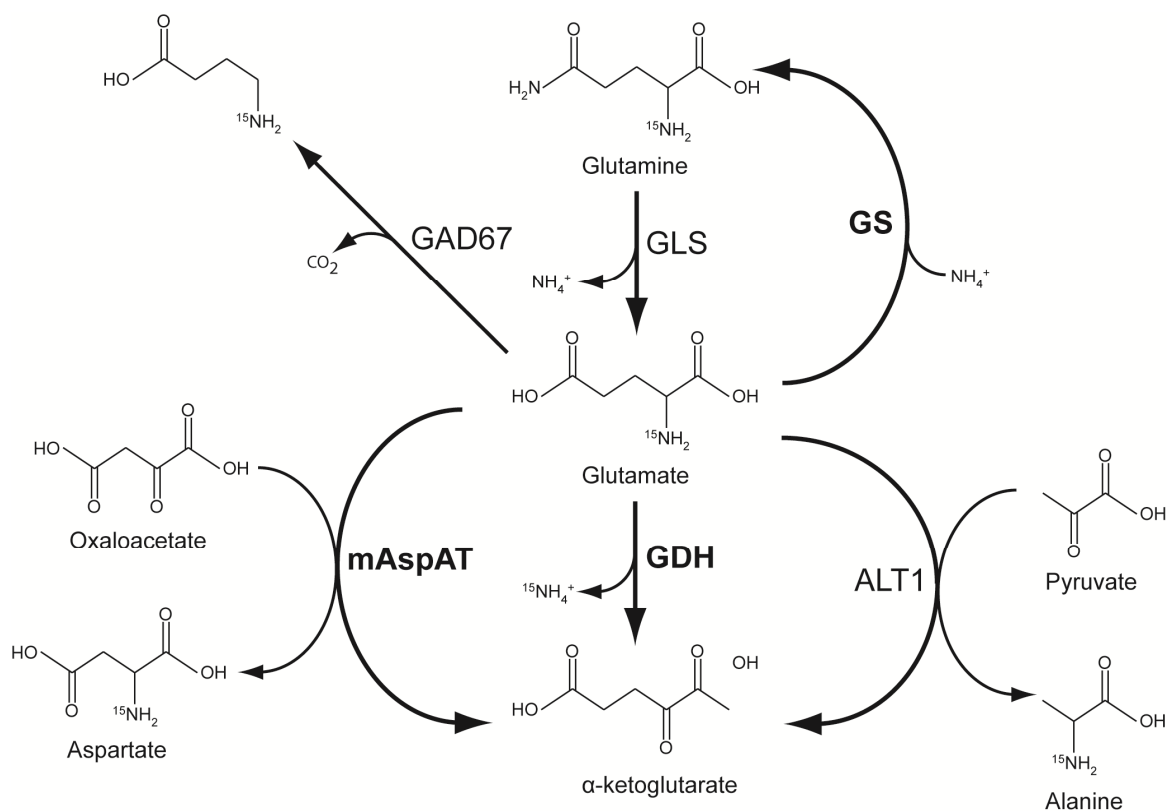


Figure 3.4. Identification of ^{15}N isotopic label in glutamate-associated metabolites. The ^{15}N isotopic label is transferred between metabolites during specific enzymatic reactions, as depicted above. Enzymes indicated in bold denote those identified as being S-nitrosylated.

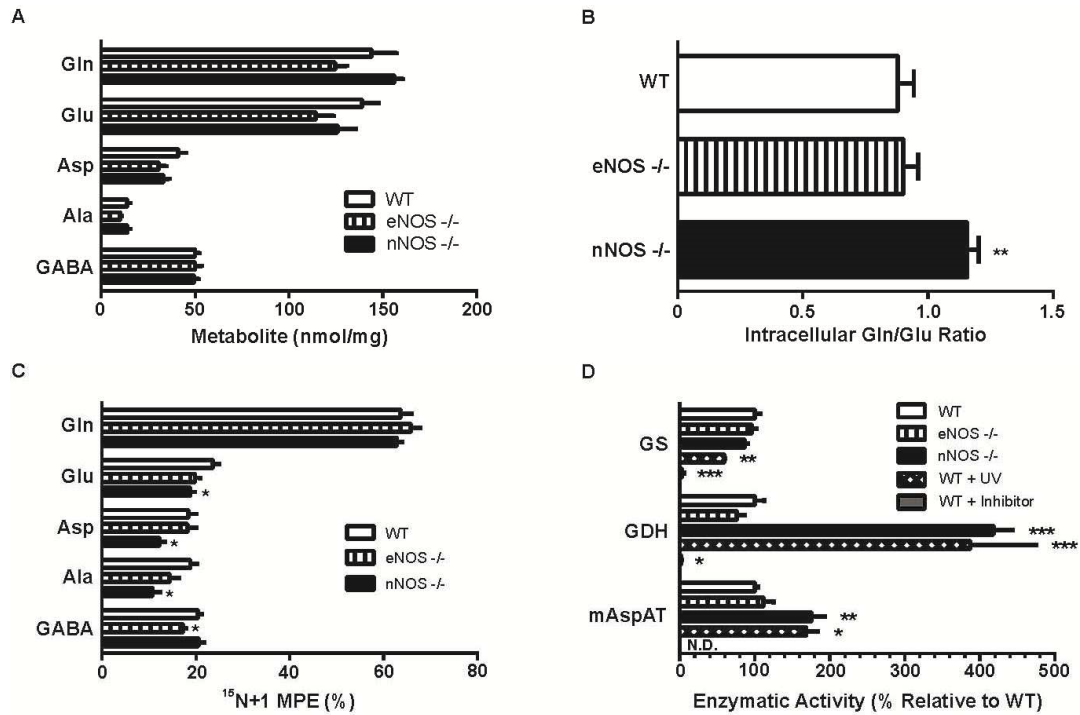


Figure 3.5. Analysis of the Glutamate/Glutamine Cycle in WT, eNOS^{-/-}, and nNOS^{-/-} Mouse Brain. **A)** Glutamate-associated metabolite quantification. Total metabolite concentrations were determined by HPLC and normalized to protein content (N = 3 mice per genotype). **B)** Intracellular glutamine/glutamate ratios. ** P<0.01 as determined by one-way ANOVA with Tukey post-hoc analysis (N = 3 mice per genotype). **C)** Genotypic differences in steady-state glutamate metabolism. ¹⁵N-based enrichment of glutamate and its associated derivatives was calculated as a percentage of total metabolite content using GC-MS and corrected for natural ¹⁵N abundance (¹⁵N+1 MPE: molar percent excess of M+1 metabolite isotopomer). * P<0.05 compared to wild-type by one-way ANOVA with Tukey post-hoc analysis between genotypes (N = 3 mice per genotype). **D)** Enzymatic activity of glutamate/glutamine effectors. Bars represent mean ± SEM (N = 3 mice per genotype), and indicate enzymatic activity relative to that measured in untreated extracts from wild-type mice. Activities in untreated wild-type extracts: GS = 1259 ± 94 nmol/mg/hr, GDH = 120 ± 15 nmol/mg/min, mAspAT = 238 ± 12 nmol/mg/min. WT + UV denotes extracts exposed to UV trans-illumination before assessing enzymatic activity. WT + Inhibitor denotes extracts pre-treated with specific enzymatic inhibitors (GS = 0.5 mM MSO, GDH = 20 μM GTP, mAspAT = 1 mM AOAA). N.D. Not detectable. *P<0.05, **P<0.01, ***P<0.001 as compared to wild-type by one-way ANOVA with Tukey post-hoc analysis.

Regulation of intracellular transport of glutamate by the S-Nitrosylation of excitatory amino acid transporter 2.

Excitatory amino acid transporter 2 is the primary transporter responsible for glutamate uptake in astrocytes of the cerebral cortex (45). Genetic deletion of the transporter results in lethal epileptic seizures in mice, and eventually leads to limited postnatal viability (46-47). Due to its importance in physiological synaptic function (48), as well as its identification in the brain S-nitrosocysteine proteome, we explored the functional consequences of excitatory amino acid transporter 2 S-nitrosylation in mouse brain synaptosomal preparations, as well as in a cell model. We quantified sodium (Na^+)-dependent glutamate uptake in freshly prepared synaptosomes from wild-type, *nNOS*^{-/-}, and *eNOS*^{-/-} forebrain in the presence or the absence of the excitatory amino acid transporter 2 inhibitor dihydrokainate (DHK) (49). The resulting difference in uptake activity between the two treatments (DHK-sensitive) is the glutamate uptake mediated by GLT1. Synaptosomal DHK-sensitive glutamate uptake was increased in *nNOS*^{-/-} synaptosomes as compared to wild-type synaptosomes (Fig. 6A). Elimination of NO from S-nitrosocysteine residues by pre-treatment of wild-type synaptosomes with copper plus ascorbate (50) increased DHK-sensitive glutamate uptake, indicating a functional regulatory role for S-nitrosylation. On the other hand, DHK-insensitive Na^+ -dependent glutamate was similar in synaptosomes prepared from the three genotypes, or in synaptosomes prepared from wild-type mice after treatment with copper and ascorbate, suggesting that non-GLT1-mediated uptake was unaffected by the altered concentrations of NO or the elimination of S-nitrosylation (Fig. 7A). We further explored the functional effect

of S-nitrosylation in HEK-293T cells that were transiently transfected with plasmids expressing either the wild-type rat transporter or a double point mutant (C373S/C562S) of the two *in vivo* S-nitrosylated cysteine residues. The cysteine residue at position 562 in the rat sequence corresponds to cysteine 561 in the mouse sequence, because rat GLT1 is one amino acid longer than the mouse protein. To induce protein S-nitrosylation, we treated cells with a non-physiological concentration of S-nitrosocysteine, which is taken up through the L-amino acid transport system. S-nitrosocysteine is an S-nitrosylating agent that can transfer an NO equivalent to reduced cysteine residues in proteins (51). As a control, cells were treated with the same concentration of cysteine. Treatment of cells with S-nitrosocysteine resulted in S-nitrosylation of the wild-type transporter (Fig. 6B), which correlated with decreased glutamate uptake (Fig. 6C). The C373S/C562S transporter was not S-nitrosylated in S-nitrosocysteine-treated cells (Fig. 6B), and its activity was similar to that of wild-type transporter and was unaffected by S-nitrosocysteine treatment (Fig. 6C). Mutation of one cysteine residue (either 373 or 562) to serine resulted in transporter activity sensitive to S-nitrosocysteine treatment, indicating that the modification of both cysteine residues is required for S-nitrosylation-mediated inhibition of glutamate transport (Fig. 7B). Relative to L-cysteine treatment, exposure of cells to S-nitrosocysteine did not alter the protein abundance of the wild-type or the C373S/C562S mutant transporter (Fig. 6D-6F), but decreased the surface abundance of both transporters by 20-25% (Fig. 6G). Kinetic analysis of GLT1-mediated glutamate uptake (Fig. 7C) revealed an 81% decrease in the K_m of the wild-type transporter after S-nitrosocysteine treatment and an 84% decrease in V_{max} . Removal of S-nitrosocysteine from the media led to a gradual decrease in

the S-nitrosylation of wild-type GLT1 that correlated with a recovery in glutamate uptake activity (Figure 6H-6I). Overall, these data suggest that specific and reversible S-nitrosylation of GLT1 at Cys³⁷³ and Cys⁵⁶² regulates the activity of the transporter.

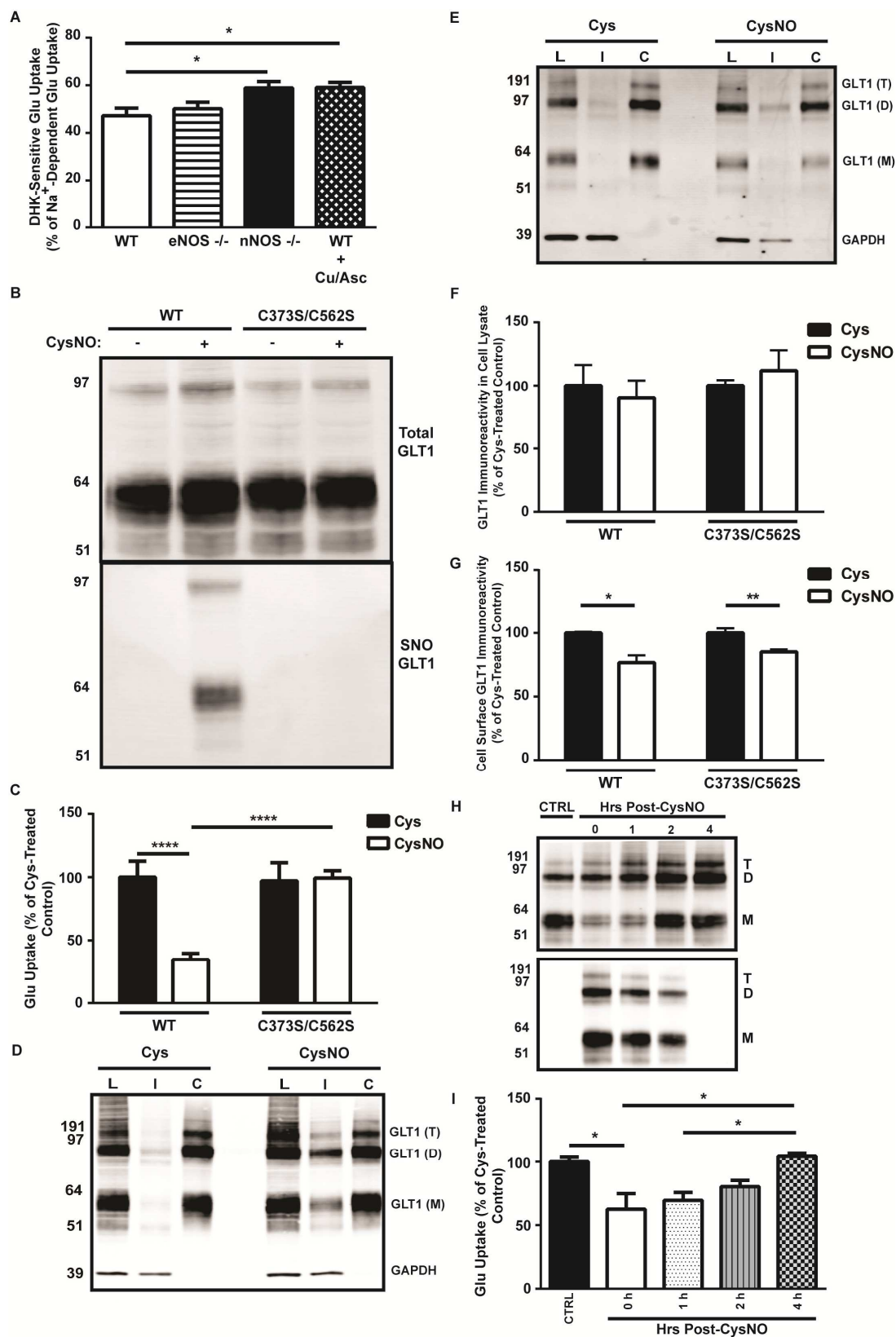


Figure 3.6. Regulation of GLT1 Function by S-Nitrosylation. All results are summarized from three mice per genotype (A) or three independent experiments in cells (B-I), and plotted as mean \pm SEM. Cells were treated with L-Cysteine (Cys) or S-nitrosocysteine (CysNO). **A)** The fraction of total Na⁺-dependent glutamate uptake that was DHK-sensitive was greater in synaptosomes from nNOS^{-/-} mice or synaptosomes pre-treated with copper and ascorbate (WT + Cu/Asc) than in untreated wild-type synaptosomes. Na⁺-dependent uptake in untreated synaptosomes of the indicated genotype: wild-type = 0.24 ± 0.03 nmol/mg/min, nNOS^{-/-} = 0.26 ± 0.06 nmol/mg/min, eNOS^{-/-} = 0.25 ± 0.04 nmol/mg/min, and WT + Cu/Asc = 0.29 ± 0.03 nmol/mg/min. *P < 0.05 after one-way ANOVA followed by Dunnett post-hoc analysis. **B-C)** S-nitrosylation of wild-type GLT1 in HEK-293T cells following CysNO treatment (B) correlated with decreased glutamate uptake (C). Cys-treated wild-type GLT1 = 0.27 ± 0.03 nmol/mg/min, Cys-treated C373S/C562S GLT1 = 0.26 ± 0.04 nmol/mg/min. ****P < 0.0001 after two-way ANOVA followed by Bonferroni post-hoc analysis. **D-E)** Representative blots from cell surface biotinylation assays of cells expressing wild-type GLT1 (D) and C373S/C562S GLT1 (E). L, lysate; I, intracellular fraction; C, cell surface fraction. 5 μ g of total lysate and equivalent dilutions of the other fractions were used. T, D, and M refer to trimeric, dimeric, and monomeric GLT1 respectively. The intracellular fraction of both forms of GLT1 was increased after CysNO treatment. **F)** Quantification of total GLT1 abundance in cell lysate. **G)** Quantification of plasma membrane abundance of GLT1 after Cys/CysNO treatment. *P<0.05, **P<0.01 as determined by ANOVA with Bonferroni post hoc analysis. **H-I)** S-nitrosylation of wild-type GLT1 was reversible (H), and correlated with a recovery in glutamate uptake (I). Wild-type GLT1 cells were exposed to Cys (CTRL) or CysNO, then to fresh media. Glutamate uptake and S-nitrosylation of GLT1 were assessed between after CysNO exposure. *P < 0.05 after one-way ANOVA followed by Bonferroni post-hoc analysis.

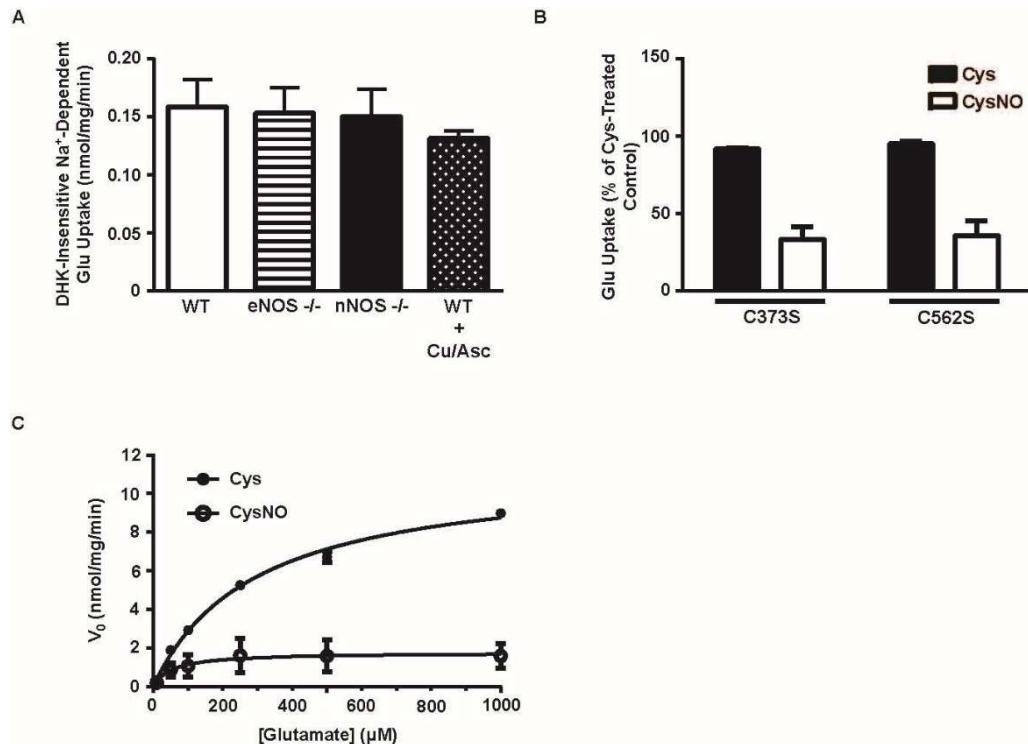


Figure 3.7. Characterization of GLT1-independent uptake in synaptosomes and GLT1-dependent uptake in cells. (A) DHK-insensitive Na⁺-dependent glutamate uptake does not differ in brain synaptosomes prepared from the three genotypes, or in synaptosomes treated with 50 μM copper plus 500 μM ascorbate, suggesting that non-GLT1-mediated uptake is unaffected by the altered concentrations of NO or the elimination of S-nitrosylation (N=3 mice per genotype). (B) Glutamate uptake is inhibited by CysNO treatment in cells expressing single-cysteine mutants of GLT1 (C373S or C562S). Cells were treated with 0.4 mM of L-Cysteine (Cys) or 0.4 mM S-nitrosocysteine (CysNO) and GLT1 mediated uptake was assessed in the presence of 5 nM ³H-glutamate. Cys-treated WT GLT1 = 0.27 ± 0.03 nmol/mg/min, Cys-treated C373S GLT1 = 0.24 ± 0.04 nmol/mg/min, Cys-treated C562S GLT1 = 0.25 ± 0.02 nmol/mg/min (N=3 independent experiments). (C) CysNO treatment of the WT transporter leads to altered kinetic parameters of GLT1-mediated glutamate uptake. A saturation curve of Na⁺-dependent glutamate uptake was determined for 10-1000 μM glutamate in cells ectopically expressing the wild-type GLT1 protein. Kinetic parameters were calculated using non-linear regression analysis of GLT1-mediated glutamate uptake and plotted as mean ± SEM (N=3 independent experiments). K_m for glutamate for Cys-treated WT GLT1 = 293 ± 27 μM, CysNO-treated WT GLT1 = 55 ± 64 μM. V_{max} for Cys-treated WT GLT1 = 11.8 ± 0.4 nmol/mg/min, CysNO-treated WT GLT1 = 1.8 ± 0.5 nmol/mg/min.

Molecular Modeling of the Effects of S-nitrosylation

We used the crystal structure of mouse mitochondrial aspartate aminotransferase (PDB 3PD6), human glutamate dehydrogenase (PDB 1L1F) and human glutamine synthetase (PDB 2QC8) as templates and generated protein structures with the specific S-nitrosylated cysteine residues. Electrostatic

potentials and partial atomic charges were calculated for S-nitrosylated and unmodified cysteine residues. All three proteins are large multimeric enzymes in which pairs of equivalent modifiable cysteine residues from different monomers are arranged with their side chains in an antiparallel fashion across from each other. In mitochondrial aspartate aminotransferase each cysteine residue at position 106 between chains A and B and C and D are 26Å apart. In glutamate dehydrogenase, each cysteine residue at position 112 between chains A and E, B & E and C & F are 20Å apart, whereas cysteine residues 99 and 183 in GS between the side chains are about 23Å apart. S-nitrosylation of the cysteine residue makes the side chain significantly more polar, increasing the dipole moment from 1.7 Debye to 5.4 Debye. This increase in polarity results in an augmented electrostatic potential which, because of the antiparallel dipole arrangement and the effective propagation of electrostatic potentials inside large proteins (52-61), produces a repulsive electrostatic 'wedge' between subunits (Fig. 8). This wedge could drive changes in the quaternary structure and provide a plausible allosteric mechanism by which S-nitrosylation of cysteine residues distant from substrate and cofactor binding sites modulates enzyme activity.

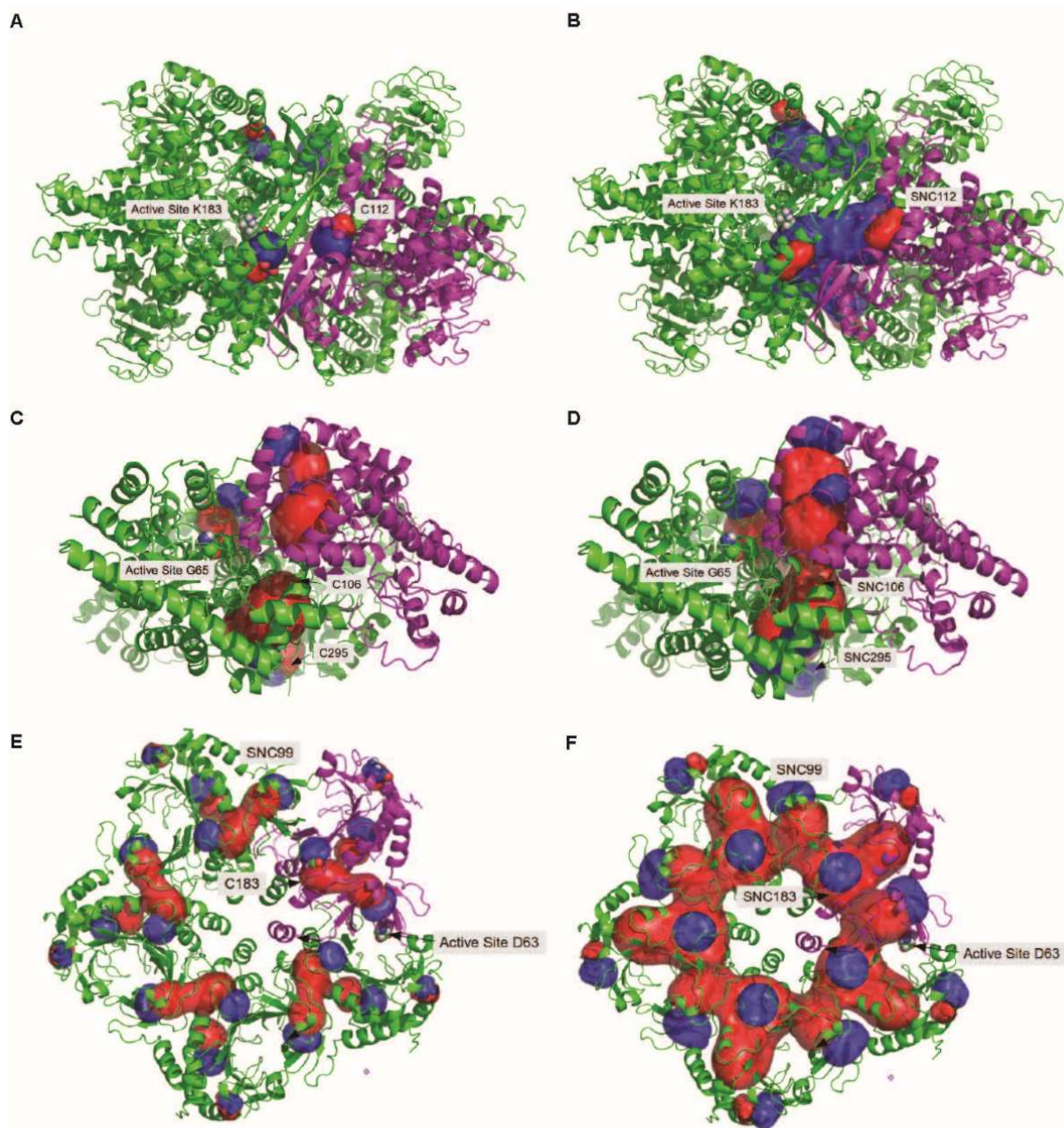


Figure 3.8. S-nitrosylation augments electrostatic potential. The crystal structure of mouse mAspAT (PDB 3PD6), human GDH (PDB 1L1F) and human GS (PDB 2QC8) were used as templates and protein structures with the specific S-nitrosylated cysteine residues were generated. Electrostatic potentials and partial atomic charges were calculated for S-nitrosylated (shown as SNC) and unmodified (shown as C) cysteine residues. Potential in GDH produced by unmodified Cys¹¹² (A) and S-nitrosyl-Cys¹¹² (B). Potential in mAspAT from Cys¹⁰⁶/Cys²⁹⁵ (C) and S-nitrosyl-Cys¹⁰⁶/S-nitrosyl-Cys²⁹⁵ (D). Potential in GS from Cys⁹⁹, Cys¹⁸³, Cys²⁶⁹, and Cys³⁴⁶ (E) and S-nitrosyl-Cys⁹⁹, S-nitrosyl-Cys¹⁸³, S-nitrosyl-Cys²⁶⁹, and S-nitrosyl-Cys³⁴⁶ (F). Isopotential contours are shown at -1kT/e in red and +1kT/e in blue. Positions of key cysteine residues, S-nitrosyl-cysteine residues, and binding site residues are indicated.

3.3 Discussion

S-nitrosylation of cysteine residues represents an alternative signaling mechanism through which nitric oxide can expand the functional diversity and biological utility of proteins. We applied a combination of chemical enrichment and mass spectrometric technologies to specifically map endogenous sites of S-nitrosylation in wild-type, *nNOS*^{-/-}, and *eNOS*^{-/-} mouse forebrain. In wild-type brain 45 sites of S-nitrosylation in 44 proteins identified by our method have been previously reported with various biochemical and proteomic approaches. The current study contributed 225 additional S-nitrosylation sites as well as 92 additional protein targets, expanding the known endogenous S-nitrosylated proteins in the mouse brain. As with any enrichment method, the identification of the endogenous sites of S-nitrosylation is limited by the relative abundance and biological stability of S-nitrosocysteine. Despite these limitations, interrogation of the proteomic data coupled with biochemical approaches provided evidence that protein S-nitrosylation participates in the coordination of glutamate metabolism and neurotransmission. Specifically, four proteins that regulate glutamate uptake, metabolism and conversion to glutamine are functionally regulated by S-nitrosylation.

Glutamate uptake through excitatory amino acid transporter 2, the major astrocytic transporter of glutamate in the CNS, was reversibly inhibited by S-nitrosylation at Cys³⁷³ and Cys⁵⁶². Irreversible alkylation of Cys³⁷³ inhibits transporter activity (62), whereas the functional role of Cys⁵⁶² in the rat or Cys⁵⁶¹ in the mouse has not been studied. Because permissive mutation of these two cysteine residues to serine did not alter excitatory amino acid transporter 2 activity, these data suggest that both cysteine residues are not critical for function

but that posttranslational modifications at these residues are important for functional regulation. We speculate that the transient inhibition of the transporter may allow for an extended period of increased glutamate concentration within the synaptic cleft, promoting neurotransmission and synaptic strengthening. The absence of nNOS-dependent S-nitrosylation of excitatory amino acid transporter 2 may prevent *nNOS*^{-/-} mice from achieving this regulation and thereby contribute to their phenotype of dysregulated glutamatergic transmission, synaptic plasticity and memory (6-14).

Metabolic studies have indicated that once in astrocytes, a fraction of glutamate (estimates vary from 50-70%) is converted to glutamine by glutamine synthetase (63-64). The remaining glutamate in astrocytes is oxidized by glutamate dehydrogenase and mitochondrial aspartate aminotransferase to α -ketoglutarate, which can then enter the TCA cycle (65-66). The metabolic demands placed on astrocytes and neurons may determine the fraction of glutamate that is oxidized in the TCA cycle. Our data indicate that S-nitrosylation inhibited glutamate oxidation by glutamate dehydrogenase and mitochondrial aspartate aminotransferase, and promoted conversion to glutamine by glutamine synthetase (Fig. 5D). Moreover, the absence of S-nitrosylation of these enzymes in *nNOS*^{-/-} mice resulted in a higher ratio of glutamine to glutamate, consistent with increased glutamate oxidation (Fig. 5B - 5C). The lower fraction of glutamate converted to glutamine may contribute to the phenotype of the *nNOS*^{-/-} mice. Although there are disagreements on the fraction of the re-cycled glutamate that participates in neurotransmission, genetic ablation of enzymes in the glutamate/glutamine cycle underscore the importance of maintaining this cycle in the CNS (67). Specifically, glutamine synthetase haploinsufficiency

leads to increased seizure susceptibility *in vivo* (68), with complete deletion of the gene associated with decreased cortical glutamine and severely limited postnatal viability (69). Glutamate dehydrogenase deletion *in vivo* leads to increased glutamine concentrations in the brain (70), whereas overexpression of the enzyme leads to decreased synaptic plasticity and age-dependent loss of synaptic and dendritic architecture (71). During periods of increased synaptic activity, the effect of S-nitrosylation on the reallocation of glutamate from oxidation to regeneration of glutamine (and subsequent glutamate) provides an explanation for how sustained neurotransmission may be achieved in wild-type mice but not in *nNOS*^{-/-} mice. The S-nitrosylation induced alteration in glutamate metabolism could also explain why *nNOS*^{-/-} mice are protected from cerebral ischemia (15). Our data suggests that neurons in the *nNOS*^{-/-} brain would avidly oxidize glutamate and consequently exhibit attenuation of the ischemia-induced increase in intra-synaptic glutamate (15-17, 72) while providing ATP for metabolic processes. The partial loss of glutamine synthetase activity in *nNOS*^{-/-} brain would hinder glutamate conversion to glutamine (an important mechanism of glutamate disposal) but would also spare limited reserves of ATP during an ischemic episode, since the glutamine synthetase pathway normally consumes considerable energy (73). Collectively, S-nitrosylation of these proteins may provide a regulatory switch between glutamate oxidation and glutamine generation to support synaptic maintenance and glutamatergic neurotransmission.

Finally, it is interesting to note that of the 136 proteins identified as targets of S-nitrosylation in the wild-type mouse brain, 23 have been identified as part of macromolecular complexes implicated in glutamate metabolism at synapses

(74). The association of glutamate uptake and mitochondrial mobility (75), the functional interaction between glutamate dehydrogenase-mediated glutamate oxidation and excitatory amino acid transporter 2-mediated glutamate uptake (76), and the data presented in figures 2 and 3 indicate that selective protein S-nitrosylation may function as a synapse-specific gatekeeper of glutamate fate during neurotransmission.

Table 3.1: S-Nitrosocysteine Sites Identified in wild-type, eNOS^{-/-}, and nNOS^{-/-} Mouse Brain

Protein name	Uniprot ID	Peptide sequence	WT	eNOS ^{-/-}	nNOS ^{-/-}
10-formyltetrahydrofolate dehydrogenase	Q8R0Y6	AVQMGMSVFFNKGENC ⁷⁰⁷ IAAGR	X		
14-3-3 protein theta	P68254	YLAEVA ^{C134} GDDRK	X		
2',3'-cyclic-nucleotide 3'-phosphodiesterase	P16330	LDEDLAGY ^{C111} RR	X		X
		LELVSYFGKRPPGVLHC ²⁵¹ TTK	X		X
2-oxoglutarate dehydrogenase E1 component, mitochondrial	Q60597	TKAEQFY ^{C395} GDTEGKK	X		
		SMT ^{C604} PSTGLEEDVLFHIGK	X	X	
		NTFHKDVVVDLVC ⁴⁹⁷ YRR	X		
4-aminobutyrate aminotransferase, mitochondrial*	P61922	TMGC ²²⁴ LATTHSK	X		
		^{C269} LEEVEDLIVKYR#	X	X	
		NKGVVLGG ^{C467} GDK	X		X
Acetyl-CoA acetyltransferase, mitochondrial	Q8QZT1	IHMGN ^{C193} AENTAK	X	X	X
Aconitate hydratase, mitochondrial	Q99K10	VAVPSTIH ^{C126} DHLIEAQVGGEK	X	X	
		^{C410} KSQFTITPGSEQIR	X	X	X
		^{C592} TTDHISAAGPWLK	X		
Actin, cytoplasmic 1*	P60710	^{C285} DVDIRKDLANTVLSGGTTMYPGI ADR	X	X	
Actin-related protein 10	Q9QZB7	^{C27} GFAGETGPR		X	
ADP/ATP translocase 1	P48962	EFNGLGD ^{C160} LTK	X		
AP-1 complex subunit beta-1	O35643	AIGRC ³⁸⁰ AIKVEQSAER	X	X	
AP-2 complex subunit alpha-1	P17426	AC ³³¹ NQLGQFLQHR	X		
AP-2 complex subunit beta	Q9DBG3	^{C129} LKDEDPYVRK	X		
		^{C380} AIKVEQSAER	X		
AP-2 complex subunit mu	P84091	SGKQSI AIDDC ²⁴⁶ TFHQ ^{C251} VR	X		
		YIGRSGIYETRC ⁴³⁵	X		
Aspartate aminotransferase, cytoplasmic	P05201	IANDNSLNHEYLPILGLAEFRS ^{C83} ASR	X		
Aspartate aminotransferase, mitochondrial*	P05202	NLDKEYLPIGGLAEFC ¹⁰⁶ K#	X		
		VGAFTVV ^{C295} K#	X		
ATP synthase subunit alpha, mitochondrial	Q03265	LY ^{C244} IYVAIGQKR	X		
ATP synthase subunit gamma, mitochondrial	Q91VR2	HLIIGVSSDRGL ^{C103} GAIHSSVAK	X	X	
ATP-citrate synthase*	Q91V92	GVTIIGPATVGGIKPG ^{C623} FK	X		

Calcium/calmodulin-dependent protein kinase type II alpha chain	P11798	C ³⁰ VKVLAGEYAAK	X	X	
		QETVDC ²⁹⁰ LKKFNAR	X		
Calcium/calmodulin-dependent protein kinase type II beta chain*	P28652	C ³¹ VKLC ³⁵ TGHEYAAK	X	X	X
		STVASMMHRQETVE ²⁹⁰ LKK#	X	X	
Citrate synthase, mitochondrial*	Q9CZU6	GYSIPE ¹⁰¹ QKMLPK#	X		X
		LP ²¹¹ VAAK	X		X
		YSC ³⁵⁹ QREFALK	X	X	
Clathrin heavy chain 1*	Q68FD5	HSSLAG ¹⁵¹ QIINYR	X		X
		AHIAQL ⁶¹⁷ EK	X		
		IHEG ⁸⁷⁰ EETHNALAK	X	X	
		YC ⁹⁰⁹ EKRDPHLA ⁹¹⁸ VAYER	X	X	
		C ¹²⁰⁵ YDEKMYDAAK	X	X	
CLIP-associating protein 2	Q8BRT1	IPRPSVSQG ⁵³⁴ SR		X	
Creatine kinase B-type	Q04447	SIRGF ¹⁴¹ LPPHC146SR	X	X	X
		FC ²⁵⁴ TGLTQIETLFK	X	X	
		LGYILT ²⁸² PSNLGTGLR	X	X	X
Creatine kinase, ubiquitous mitochondrial*	P30275	KHNN ⁶³ MASHLTPAVYAR	X	X	X
		LGYILT ³¹⁷ PSNLGTGLR	X	X	X
Cullin-associated NEDD8-dissociated protein 1	Q6ZQ38	LDRLVEPLRAT ¹¹⁵³ TTK	X		
Cysteine and glycine-rich protein 1	P97315	TVYFAEEVQC ²⁵ EGNSFHK	X		
		NLDSTTVAVHGEEIYC ⁵⁸ KSC ⁶¹ YGKK	X		
		C ¹²² SQAVYAAEK	X	X	
Cysteine-rich protein 2	Q9DCT8	C ⁸ DKTVYFAEK	X	X	X
Cytochrome b-c1 complex subunit 6, mitochondrial*	P99028	ERLEL ⁵¹ DNRVSSR	X	X	
		DHC ⁷⁹ VAHKLFK	X		
Cytochrome c oxidase subunit 5B, mitochondrial*	P19536	C ¹¹² PNC ¹¹⁵ GTHYKLVPHQMAH#	X	X	X
Cytochrome c, somatic	P62897	IFVQKC ¹⁵ AQCHTVEKGGK	X		
Cytochrome c1, heme protein, mitochondrial	Q9D0M3	ARHGGEYVFSLLTGYC ²¹⁹ EPPTGVS LR	X		
Cytosolic 5'-nucleotidase III-like protein	Q3UFY7	C ⁴⁸ PSSHNILDNSK	X		
		AHSLC ¹⁰⁶ QQR	X		
D-3-phosphoglycerate dehydrogenase	Q61753	ALVDHENVISC ²⁸¹ PHLGASTK	X	X	X

D-beta-hydroxybutyrate dehydrogenase, mitochondrial	Q80XN0	GFLVFAGC ⁸⁶ LMK	X		
		SPYC ²⁰⁹ ITK	X		
		TIQLNV ¹¹⁵ NSEEEKAVETIR	X		
D-dopachrome decarboxylase	O35215	LC ²⁴ AATATILDKPEDR	X		
Destrin	Q9R0P5	C ²³ STPEEIK	X	X	
		AVIFC ³⁹ LSADKK	X	X	
Dihydrolipoyl dehydrogenase, mitochondrial*	O08749	VC ⁴⁸⁴ HAHPTLSEAFR	X	X	
Dihydropyrimidinase-related protein 1	P97427	SHKSTVEYNIFEGMEC ⁴³⁹ HGSPLVVIS QGK	X		
Dihydropyrimidinase-related protein 2*	O08553	SITIANQTN ²⁴⁸ PLYVTK#	X	X	
		THNSALEYNIFEGMEC ⁴³⁹ R#	X	X	
		GVPRGLYDGPVC ⁵⁰⁴ EVSVTPK#	X	X	
Dihydropyrimidinase-related protein 3	Q62188	FIPC ⁴⁷¹ SPFSDYVYKR	X	X	
Dihydropyrimidinase-related protein 5	Q9EQF6	DSELYQVFHAC ¹⁸¹ R	X	X	
		C ⁴³² HGVPLVTISR	X	X	
DmX-like protein 2*	Q8BPN8	HGNIQVSC ⁶² VEC ⁶⁵ SNQHGR	X		
		C ¹³⁷³ IAGEVAIVRDPDAGEGTR	X		
		VINLSQYGPA ¹⁵⁰⁴ FGQEHAR#	X		
		LHTC ¹⁵⁷⁷ LLTSLPLYR	X		
		SC ¹⁸³² NPVVF ¹⁵⁷⁷ SYNYLR	X		
		VGC ¹⁸⁹⁶ PVLALEVL ¹⁵⁷⁷ SKIPK	X		
Dpys protein (Fragment)	Q8K0X3	C ⁴³³ HGVPLVTISR			X
Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial	Q921G7	VTVFAEGC ²⁴⁷ HGHLAK	X		
		LQINAQNC ⁵⁸⁵ VHC ⁵⁸⁸ K	X		
Elongation factor Tu, mitochondrial	Q8BFR5	KGDEC ²⁹⁰ ELLGHNK	X		
ES1 protein homolog, mitochondrial	Q9D172	KPIGLC ¹⁷⁴ C ¹⁷⁵ IAPVLA ¹⁷⁵ AK	X	X	
		HC ²¹⁹ VKGVTEAHVDQK	X	X	
		NKVVTTPAFMC ²⁴² ETALHHIHDGIGAM VK	X	X	
Excitatory amino acid transporter 2	P43006	C ³⁷³ LEDNLGIDKR	X	X	
		SADC ⁵⁶¹ SVEEPEWKR	X	X	
Fascin (Fragment)	D3YWW ₃	LINRPIIVFRGEHGFIC ³⁹⁷ R			X
Fatty acid synthase*	P19096	C ¹⁰⁸⁴ LGITVSGGIHIS ¹⁰⁸⁴ R	X		
		C ¹⁴⁶⁴ ILLSNL ¹⁴⁶⁴ SNTSHAPK	X		

Fructose-bisphosphate aldolase A	P05064	QLLLTADDRVNP ^{C73} IGGVILFHETLYQK	X		X
		^{C150} VLKIGEHTPSALAIMENANVLAR	X		
		YASIC ¹⁷⁸ QQNGIVPIVEPEILPDGDHDLKR	X		
		^{C202} QYVTEK	X		X
		ALANSLA ^{C339} QGK	X	X	
Fructose-bisphosphate aldolase C	P05063	K ^{C73} IGGVIFFHETLYQKDDNGVPFVR	X		
		^{C150} VLKISDRTPSALAILANANVLAR	X		
		YASIC ¹⁷⁸ QQNGIVPIVEPEILPDGDHDLKR	X		
		^{C202} QYVTEKVLAAVYK	X		
Fructose-bisphosphate aldolase	Q9CPQ9	QLLLTADDRVNP ^{C73} IGGVILFHETLYEK	X	X	X
		^{C202} QYVTEKVLAAVYK	X	X	X
		ALANSLA ^{C339} QGK	X	X	X
Galectin-related protein A	Q8VED9	LIVPFC ⁴¹ GHIK		X	
Glutamate decarboxylase 1	P48318	LGLKIC ⁴⁷ GFLQR	X	X	
		QSSKNLLSC ⁷⁸ ENSDQGAR	X	X	
Glutamate dehydrogenase 1, mitochondrial	P26443	IIKPC ¹¹² NHVLSLSFPIR	X	X	
Glutamine synthetase*	P15105	LVL ^{C99} EVFK	X		X
		AC ¹⁸³ LYAGVK	X	X	
		^{C269} IEEAIDKLSK#	X	X	
		RPSAN ^{C346} DPYAVTEAIVR#	X	X	
Glyceraldehyde-3-phosphate dehydrogenase*	P16858	IVSNASC ¹⁵⁰ TTNC ¹⁵⁴ LAPLAK#	X	X	
		VPTPNVSVVDLT ^{C245} R	X	X	
Glycerol-3-phosphate dehydrogenase, mitochondrial	Q64521	^{C270} KDVLTGQEFDVR	X	X	
		^{C285} VINASGPFTDSVR	X	X	
Glycogen phosphorylase, brain form*	Q8CI94	FGC ³¹⁹ RDPVR	X		X
		QLLNC ⁵⁸¹ LHIITLYNR	X		X
Guanine nucleotide-binding protein G(i), alpha-2 subunit	P08752	EIYTHFTC ³²⁶ ATDTK	X		
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1*	P62874	KAC ²⁵ ADATLSQITNNIDPVGR#	X	X	
		ELAGHTGYLSC ¹⁴⁸ C ¹⁴⁹ R#	X	X	X
		LFVSGAC ²⁰⁴ DASAK#	X	X	
		ADQELMTYSHDNII ^{C271} GITSVSFSK	X	X	
Heat shock protein 105 kDa	Q61699	FIC ⁶⁵⁹ EQEHEK	X	X	
		VKELNNVC ⁷⁹⁷ EPVVTQPKPK	X	X	

Hexokinase-1*	P17710	ATDC ⁶⁸⁴ VGHDTVATLLR	X		X
		KLPVGFTFSFP ^{C214} R	X		X
Histidine triad nucleotide-binding protein 1	P70349	^{C84} AADLGLKR	X	X	
Huntingtin	P42859	LRGGGGNVTLGE ^{C1706} SEGK	X		
		^{C1940} ENLSTPTTLKK	X		
Hypoxanthine-guanine phosphoribosyltransferase	P00493	DLNHV ^{C206} VISETGK	X		
Inositol monophosphatase	O55023	LC ¹⁸³ SIPIHGIR	X	X	
Inositol-3-phosphate synthase 1	Q9JHU9	SSVDDMVHSHVLYAPGERPDH ^{C38} ⁰ VVIK	X	X	
Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial*	Q9D6R2	KTFDLYANVRP ^{C49} VSIEGYK	X		
		DLGGNAKC351SDFTEEIC ³⁵⁹ RR#	X		
Isocitrate dehydrogenase [NAD] subunit gamma, mitochondrial	P70404	TSLDLYANVIH ^{C148} K	X	X	X
		NIANPTATLLASC ³³³ MMLDHLK	X	X	X
Isoform 11 of Myelin basic protein	P04370-11	SRPGL ^{C79} HMYKDSHTR		X	
Isoform M1 of Pyruvate kinase isozymes M1/M2	P52480	NTGII ^{C49} TIGPASR	X		
		AGKPIV ^{C326} ATQMLESNIK	X		
		^{C423} LAAALIVLTESGRSAHQVAR	X		
L-asparaginase	Q8C0M9	^{C114} IANPVKLAR	X	X	
		TPH ^{C132} FLTGHGAEK	X	X	
LIM and SH3 domain protein 1	Q61792	KPY ^{C53} NAHYPK	X	X	X
L-lactate dehydrogenase A chain	P06151	IVSSKDY ^{C84} VTANSK	X	X	
		VIGSG ^{C163} NLDSAR	X	X	
L-lactate dehydrogenase B chain	P16125	HRVIGSG ^{C164} NLDSAR	X	X	
Long-chain-fatty-acid--CoA ligase 6	Q91WC3	VIQSVVY ^{C336} HGGR	X	X	
Malate dehydrogenase, cytoplasmic*	P14152	VIVVGNPANTN ^{C137} LTASK#	X		X
		SAPSIPKENFSC ¹⁵⁴ LTR#	X	X	X
Malate dehydrogenase, mitochondrial	P08249	VNVPVIGGHAGKTIIPILISQ ^{C212} TPK	X	X	X
		ETE ^{C285} TYFSTPLLLGKK	X		
Microtubule-associated protein 1A*	Q9QYR6	SIEEAC ¹⁶⁰ LTQLHLNR	X	X	
		SAP ^{C2165} GSLAFSGDR#	X	X	
Microtubule-associated protein 1B	P14873	SALRDAY ^{C1224} SEEKELK	X	X	
		TIKSP ^{C1913} DSGYSETIEK	X		

Microtubule-associated protein 2	P20357	YTRPTHLS ^{C1506} VK	X		
		KIDLSHVTSK ^{C1709} GSLK	X		
Microtubule-associated protein RP/EB family member 3	Q6PER3	LSNVAPP ^{C182} ILRK	X		X
Microtubule-associated protein tau	P10637	^{C614} GSLGNIHHKPGGGQVEVK	X	X	
Mitochondrial carrier homolog 2	Q791V5	QVC ^{C49} QLPGLFC ^{C56} YAQHIASIDGRR	X	X	
		LC ^{C79} SGVLGTVVHGK	X	X	X
Mitochondrial glutamate carrier 1	Q9D6M3	GVNEDTYSGLD ^{C271} ARK	X		
Myelin proteolipid protein*	P60202	TTIC ^{C109} GKGLSATVTGGQK	X	X	X
		TSASIGSL ^{C201} ADAR#	X	X	X
NAD-dependent protein deacetylase sirtuin-2	Q8VDQ8	^{C164} YTQNIDTLER	X		X
NADH dehydrogenase [ubiquinone] iron-sulfur protein 6, mitochondrial	P52503	IIAC ^{C79} DGGGGALGHPK	X	X	X
		TGTC ^{C104} GYCGLQFKQH HH	X	X	
Non-POU domain-containing octamer-binding protein	Q99K48	FAC ^{C147} HSASLTVR	X	X	X
Peptidyl-prolyl cis-trans isomerase A*	P17742	IIPGFM ^{C62} QGGDFTR	X	X	X
		HTGPGILSMANAGPNTNGSQFFI ^{C115} TAK	X		
		KITISD ^{C161} GQL#	X	X	X
Peroxisome oxidin-5, mitochondrial*	P99029	KGVLFVGPAGFTPG ^{C96} SK	X		
Phytanoyl-CoA hydroxylase-interacting-like protein	Q8BGT8	KDPSC ^{C321} KT ^{C324} NISVGR	X	X	
Plexin-A1	P70206	IQSC ^{C374} YRGEK	X		X
		GYEC ^{C742} LFHIPGSPAR	X	X	X
		^{C1011} LTPPGHTPGSAPIVINIR	X	X	X
Profilin-1	P62962	^{C128} YEMASHLR	X	X	
Protein DJ-1	Q99LX0	VTVAGLAGKDPVQC ^{C46} SR	X	X	
		KGLIAAI ^{C106} AGPTALLAHEVGFG ^{C121} K	X	X	
Protein kinase C and casein kinase substrate in neurons protein 1	Q61644	AYHLA ^{C161} KEER	X	X	
Protein kinase C beta type*	P68404	^{C132} DT ^{C135} MMNVHKK	X	X	
		^{C143} VMNVPSL ^{C151} GTDHTERR	X	X	
Protein phosphatase 1 regulatory subunit 1B	Q60829	RPNP ^{C72} AYTPPSLK	X		
Putative uncharacterized protein	Q3ULN6	SVSC ^{C218} LKPVPIVGTK			X
Pyridoxal kinase	Q8K183	^{C273} AKAEAGEGQKPSPAQLELR	X	X	

Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial*	P35486	C ⁴¹ DLHRLEEGPPVTTVLTR	X	X	X
		EATKFAAAAYC ²⁷³ R	X	X	X
Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	Q9D051	QGTHITVVAHSRPPVGH C ²⁴⁹ LEAAAVL SK	X		
Pyruvate kinase isozymes M1/M2	P52480	NTGII C ⁴⁹ TIGPASR	X	X	X
		AGKPVI C ³²⁶ ATQMLESNIK	X	X	
Rab GDP dissociation inhibitor alpha	P50396	IIC ³⁰² ILSHPIK	X	X	X
		KSDIYV C ³³⁵ MISFAHNVAAGK	X	X	X
Rab GDP dissociation inhibitor beta	Q61598	VGQVIRVI C ³⁰² ILSHPIK	X	X	
		KSDIYV C ³³⁵ MISFAHNVAAGK	X	X	X
Ras-related C3 botulinum toxin substrate 1	P63001	HH C ¹⁰⁵ PNTPIILVGTK	X	X	X
		AVL C ¹⁷⁸ PPPVKK	X	X	X
Ras-related protein Rab-14	Q91V41	SC ²⁶ LLHQFTEK	X	X	
		FMAD C ⁴⁰ PHTIGVEFGTR	X	X	
Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	O55143	SMSVY C ⁴⁹⁸ TPNKPSR	X		
		GAPEGVIDR C ⁵²⁴ THIR	X		
		C ⁵⁶⁰ LALATHDNPLKR	X		
		NYLEQPGKE C ⁹⁹⁸ VQPATK	X	X	X
Secernin-1	Q9CZC8	C ¹⁸⁶ IC ¹⁸⁸ NHLSLATKLDEEHPELR	X	X	X
		SSP C ²⁹⁰ IHYFTGTPDPSR	X	X	X
Septin-5	Q9Z2Q6	DVT C ³⁰⁰ DVHYENYR	X	X	X
Serum albumin	P07724	LQT C ³⁰² C ³⁰³ DKPLKK	X		
S-methyl-5'-thioadenosine phosphorylase	Q9CQ65	TSLRPQTFYDGSH C ¹³⁰ SAR	X		
		C ¹⁶³ HSKGTIVTIEGPR	X		
Sodium- and chloride-dependent GABA transporter 3	P31650	TVT VND C ⁶⁰⁷ EAKVKGDGTISAITEK	X		
Sodium/potassium-transporting ATPase subunit alpha-1	Q8VDN2	C ⁵¹⁸ SSILLHGK	X	X	X
		AAVPDAVGK C ⁶⁰⁶ R	X		
		DAKAC ⁶⁶³ VVHGSDLK	X		
		LIIVEG C ⁷⁰⁵ QRQGAIVAVTGDGVNDSP ALKK	X		

Sodium/potassium-transporting ATPase subunit alpha-2*	Q6PIE5	IISSHG ^{C209} KVDNSSLTGESEPTQR	X	X	
		ILDR ^{C515} STILVQ GK	X	X	
		VLGF ^{C553} QLNLPSGKFPR	X		X
		AAVPAVGK ^{C603} R	X		
		LIIVEG ^{C702} QRQGAIVAVTGDGVNDSP ALKK#	X		
Sodium/potassium-transporting ATPase subunit alpha-3*	Q6PIC6	YNTD ^{C49} VQGLTHSKAQEILAR#	X	X	X
		IISAHG ^{C201} KVDNSSLTGESEPTQR#	X	X	X
		SPDC ^{C221} THDNPLETR	X	X	
		DVAGDASESALLK ^{C449} IELSSGSVK#	X		
		ILDR ^{C508} ATILLQ GK#	X		
		VLGF ^{C546} HYLPEEQFPK	X		
		AAVPAVGK ^{C596} R	X		X
		AC ^{C653} VIHGTDLK#	X		
		LIIVEG ^{C695} QRQGAIVAVTGDGVNDSP ALKK#	X		
Sodium/potassium-transporting ATPase subunit beta-1*	P14094	YNPNVLPVQC ^{C214} TGKR#	X		
Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	Q8K2B3	TYFSC ^{C266} TSHTSTGDGTAMVTR	X		
		AC ^{C467} ALSIAESC ^{C475} RP GDKVPSIK	X	X	X
Superoxide dismutase [Cu-Zn]*	P08228	AVC ^{C7} VLKGDGPVQGTIHFEQK#	X	X	
Synaptophysin*	Q62277	LHQVYFDAPS ^{C87} VK#	X		
Syntaxin-binding protein 1	O08599	AAHVFFTDSC ^{C110} PDALFNELVK	X	X	
		LAEQIATLC ^{C180} ATLKEYPAVR	X	X	
TAR DNA-binding protein 43	E9Q830	YRNPVSQC ^{C50} MR		X	
Transketolase	P40142	DRTVPFC ^{C386} STFAAFFTR	X		X
Tubulin alpha-1A chain*	P68369	AYHEQLSVAEITNAC ^{C295} FEPANQMVK #	X		
		YMAC ^{C315} C ^{C316} LLYRGDVVPK	X	X	
		TIQFVDWC ^{C347} PTGFK	X		
Tubulin alpha-1B chain	P05213	C ^{C305} DPRHGKYMAC ^{C315} C ^{C316} LLYR	X	X	X
Tubulin alpha-4A chain*	P68368	C ^{C305} DPRHGKYMAC ^{C315} C ^{C316} LLYR	X	X	
		SIQFVDWC ^{C347} PTGFK#	X		X
Tubulin beta-2A chain	Q7TMM9	MREIVHIQAGQC ^{C12} GNQIGAK	X		
		NMMAAC ^{C303} DPRHGRLTVAAIFR	X		
		TAVC ^{C354} DIPPRGLK	X		
Tubulin beta-2B chain*	Q9CWF2	EIVHIQAGQC ^{C12} GNQIGAK	X		X
		NMMAAC ^{C303} DPRHGR	X		X

Tubulin beta-2C chain*	P68372	EIVHIQAGQC ¹² GNQIGAK#	X		
		TLKLTTPTYGDLNHLVSATMSGVTT ³⁹ C ² LR	X		
		NMMAAC ³⁰³ DPRHGRLTVAAVFR	X	X	X
		TAVC ³⁵⁴ DIPPRGLK	X		
Tubulin beta-3 chain	Q9ERD7	MREIVHIQAGQC ¹² GNQIGAK	X		
		VAVC ³⁵⁴ DIPPRGLK	X		
Tubulin beta-4 chain*	Q9D6F9	EIVHLQAGQC ¹² GNQIGAK#	X		
Tubulin beta-5 chain	P99024	MREIVHIQAGQC ¹² GNQIGAK	X		
		NMMAAC ³⁰³ DPRHGRLTVAAVFR	X		
		TAVC ³⁵⁴ DIPPRGLK	X		
Tubulin polymerization-promoting protein	Q7TQD2	LC ⁷⁹ KDC ⁸² HVIDGK	X	X	
Ubiquitin carboxyl-terminal hydrolase isozyme L1*	Q9R0P9	NEAIQAAHDSVAQEGQC ¹⁵² R	X		
		FSAVALC ²²⁰ K	X	X	X
Ubiquitin thioesterase OTUB1	Q7TQI3	TRPDGNC ⁹¹ FYR	X	X	
Ubiquitin-conjugating enzyme E2 O	Q6ZPJ3	LG ³³⁶ FDHAQR	X		
		C ³⁴⁸ LYVFPAKVEPAK	X	X	
Ubiquitin-like modifier-activating enzyme 1	Q02053	YSRPAQLHIGFQALHQFC ³⁴⁰ ALHNQP PRPR	X		
		C ⁵⁸⁸ VYYRKPLLESGLTGTK	X	X	
Uncharacterized protein	E9QM90	LSPAFHQALLSFC ⁴⁹⁷ R	X		
		C ⁵²⁶ LPHIVPNVLLAK	X		
Vesicle-fusing ATPase*	P46460	C ¹¹ PTDELSLSNC ²¹ AVVNEKDFQSGQ HVMVR	X		
		THPSVVPGC ⁵⁹ IAFSLPQRK	X	X	
		GILLYGPPGC ²⁶⁴ GK	X		X
Voltage-dependent anion-selective channel protein 3*	Q60931	YKVC ⁶⁵ NYGLTFTQK#	X	X	
		YKLD ²²⁹ RTSLSAK	X		
V-type proton ATPase subunit B, brain isoform	P62814	KTSC ¹¹² EFTGDILR	X		
		GQKIPIFSAAGLPHEIAAQIC ²⁰⁷ R	X		
Zinc transporter ZIP6	Q8C145	KAF ¹⁵³ PDLDSDNSGKNPR	X	X	
		AC ³¹² LIHTASEK	X	X	

X denotes identification of peptide in a given genotype. The peptides corresponding to wild-type mice have been identified in at least three biological replicates.

* denotes proteins identified in previously published studies as endogenously S-nitrosylated in mouse brain

denotes peptides identified in previously published studies as endogenously S-nitrosylated in mouse brain

3.4 Materials and Methods

Chemicals and Reagents

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated. Rabbit polyclonal antibody against GLT1 was a gift from Dr. Jeffrey Rothstein (Johns Hopkins University School of Medicine, Baltimore, MD). Goat polyclonal antibodies against mAspAT and GDH were purchased from Abcam (Cambridge, MA). Mouse monoclonal antibody against GS was purchased from Millipore (Bedford, MA). L-[3,4-³H] glutamic acid was purchased from Perkin-Elmer (Waltham, MA). Affigel-10 was purchased from Bio-Rad (Hercules, CA). EZLink Sulfo-NHS-Biotin and UltraLink monomeric avidin beads were purchased from Pierce (Rockford, IL).

Animals

All procedures were performed in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Children's Hospital of Philadelphia Animal Care and Use Committee. Male mice between 10-12 weeks old were used for proteomic studies, enzymatic assays and synaptosomal uptake, while those between 8-10 weeks old were used for ¹⁵N stable isotopic profiling. Wild-type C57BL/6J (#000664), *Nos1^{tm1Unc}* C57BL/6J (*nNOS*^{-/-}: #002986), and *Nos3^{tm1Unc}* C57BL/6J (*eNOS*^{-/-}: #002684) mice were obtained from Jackson Laboratories (Bar Harbor, ME). For experiments except those involving isotopic profiling, mice were anesthetized by CO₂ and perfused through the left ventricle with ice-cold PBS. Intact organs were collected, immediately frozen in liquid nitrogen, and stored at -80 °C until use.

Identification of Protein S-Nitrosocysteine Sites

A detailed experimental procedure for the preparation and activation of columns, as well homogenate preparation for reaction with organic mercury resin, has been previously published (30). Six biological replicates from each genotype were analyzed. Each sample had a corresponding UV-pretreated negative control analyzed under identical conditions. The number of peptides identified in both untreated and UV-pretreated homogenates was used to calculate the false identification rate (FIR). The FIR was calculated independently for each biological replicate and the average value is reported. Overall, the FIR across the six biological replicates was 5.2 ± 0.7 %, which is within the range of FIR reported for site specific identification of other post-translational modifications using enrichment approaches (77-78). Columns were initially washed with 50 bed volumes of 50 mM Tris-HCl, pH 7.4, containing 0.3 M NaCl, 0.5% SDS, followed by 50 bed volumes of the same buffer containing 0.05% SDS. Columns were then washed with 50 bed volumes of 50 mM Tris-HCl pH 7.4, containing 0.3 M NaCl, 1% Triton X-100, 1M Urea. This was followed by 50 bed volumes of the same buffer containing 0.1% Triton X-100, 0.1 M urea. Finally, columns were washed with 200 bed volumes of water before proteins were eluted with 10 mL of 50 mM β -mercaptoethanol in water. Samples were concentrated and resolved by one-dimensional SDS-PAGE, followed by either western blot analysis or in-gel trypsinization and subsequent LC-MS/MS analysis (28-30, 79).

For quantification of S-nitrosylated GLT1, GDH, mAspAT, and GS from wild-type mouse brain, the same protein capture protocol described above was followed. The eluted bound proteins, representing the S-nitrosylated fraction, 30 μ g of input homogenate (total protein) and different dilutions of recombinant

standard proteins were resolved by one-dimensional SDS-PAGE and transferred to Immobilon-FL PVDF membranes (Millipore, Bedford, MA). After probing with appropriate antibodies, blots were scanned by the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).

To identify sites of S-nitrosocysteines, columns were washed with 10 bed volumes of 0.1 M ammonium bicarbonate after the final wash with water in the previously described method. Bound proteins were subjected to digestion by the addition of 1 μ g/mL of trypsin gold (Promega, Madison, WI) in one bed volume of 0.1 M ammonium in the dark for 16 hours at room temperature. The resin was next washed with 40 bed volumes of 1 M ammonium bicarbonate, pH 7.4, containing 300 mM NaCl, followed by 40 volumes of the same buffer without NaCl. Columns were then washed with 40 volumes of 0.1 M ammonium bicarbonate followed by 200 volumes of deionized water. Performic acid was synthesized by reacting 1% formic acid and 0.5% hydrogen peroxide for at least 60 minutes at room temperature (with rocking) in a glass vial shielded from light. To elute bound peptides, the resin was incubated with one bed volume of performic acid in water for 30 minutes at room temperature (28-30). Eluted peptides were recovered by washing the resin with one bed volume of deionized water. Eluates were stored at -80 °C overnight followed by lyophilization and re-suspension into 300 μ L of 0.1% formic acid. Peptide suspensions were transferred to low retention tubes (Axygen, Union City, CA) and the volume was reduced to 30 μ L by speed vacuum. Twenty μ L of peptide suspension was transferred to an HPLC vial and submitted for LC-MS/MS analysis. The details for MS/MS analysis have been provided previously (28-30, 79). Post-MS analysis to generate the S-nitrosocysteine proteomes (Table S1) was performed

as described previously (28-30). The peptides that are reported for the wild-type mice had been identified in at least three biological replicates.

Gene Ontology Analysis

Proteins in supplemental table S1 were mapped to UniprotIDs and only reviewed, non-fragment proteins were retained in the final proteome. A mouse brain reference brain proteome consisting of 7025 proteins was generated from the literature (31-34) and our experimental data. For gene ontology (GO) analysis, the Ratio of Enrichment (R) score was calculated by WebGestalt (35) as the ratio (O/E) of the observed number (O) of proteins with a specific GO annotation in the S-nitrosocysteine proteome vs. the number of annotated proteins expected (E) to be in the S-nitrosocysteine proteome. E is derived from the number of proteins annotated as part of a specific molecular pathway in the whole brain proteome normalized to the number of total proteins in the whole brain protein proteome (and multiplied by the total number of S-nitrosylated proteins). Significant enrichment was evaluated at an adjusted cutoff of 0.01, with the top ten enriched pathways considered for further analysis.

Glutamate/Glutamine Metabolism Studies

For isotopic profiling studies, three mice per genotype were analyzed. Each day samples from one mouse per genotype were prepared and analyzed. Animals were anesthetized with isoflurane and decapitated. The brain was then rapidly removed and coronal hippocampal slices (400 μ m) cut with a vibratome (*Vibratome 1000 Plus, Vibratome*, St. Louis, MO) in an ice-cold artificial cerebrospinal fluid solution (ACSF) of the following composition: 202 mM sucrose, 3 mM KCl, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 10 mM glucose, 1 mM MgCl₂, 2 mM CaCl₂, pH 7.2–7.4 (when saturated with 95% O₂-5% CO₂). Slices

were then equilibrated for 2 hours at 34°C in ACSF containing 130 mM NaCl instead of sucrose. Following equilibration, hippocampal slices were transferred into cell culture inserts and incubated in a 24-well plate for 45 minutes at 34°C in 1 mL depolarizing artificial cerebrospinal fluid (dACSF) containing the following: 130 mM NaCl, 3 mM KCl, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 10 mM glucose, 2 mM CaCl₂, 1 mM [2-¹⁵N] L-glutamine, and 0.3 mM NH₄Cl. Medium was then collected from each sample and frozen at -80°C, and slices were washed with 2 mL chilled D-PBS. Slices were homogenized in 4% perchloric acid and subjected to two freeze/thaw cycles, with a subsequent centrifugation step at 10000 x g for 15 minutes. Supernatant was collected from each slice, which contained the metabolites of interest, and protein pellets were separately resuspended in 1 M NaOH. Protein content per slice was determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Supernatant from each sample was neutralized with KOH and used for metabolite determination and measurement of ¹⁵N enrichment by GC-MS (40-41).

Enzymatic Assays

For enzymatic activities three mice per genotype were analyzed. Each day, three mice (one mouse per genotype) were analyzed. Enzymatic activities were quantified in triplicates with technical reproducibility greater than 90%. Following, the average raw values were normalized to those of untreated wild-type homogenate analyzed in the same day.

For glutamine synthetase activity, frozen tissue from mouse brain cortex were processed as described previously (80) with minor modifications. Briefly, cortices were homogenized in 5 volumes of 0.1 M imidazole pH 7.2 using an Ultra-Turax homogenizer. Homogenates were centrifuged at 10000 x g for 10

min at 4°C, and the clarified supernatant used for assessing glutamine synthetase activity (with protein concentration determined using the Bio-Rad Protein Assay). For UV light exposure to eliminate NO from S-nitrosocysteine, part of each sample from wild-type mice was trans-illuminated by UV light while on ice for 3 minutes. Activity was determined in triplicate using 90 µg of clarified homogenate in a total volume of 70 µL of reaction buffer (1 mM L-glutamate, 20 mM ATP, 40 mM MgCl₂, 0.1 M hydroxylamine, 0.1 M imidazole pH 7.2), after incubation for 20 minutes at 37°C. The reaction was stopped by the addition of 190 µL of 0.37 M FeCl₃ in 0.67 M HCl/0.2 M TCA, and samples were incubated on ice for 5 minutes. Sample mixtures were then centrifuged at 4 °C for 5 minutes at 10000 x g, and the absorbance of the resulting supernatant was measured at 535 nm and compared to a standard curve of authentic L-glutamylhydroxamate. GS activity was expressed as nmol of L-glutamylhydroxamate per mg of protein per hour. Enzymatic specificity of the assay was determined using 0.5 mM L-methionine sulfoximine (MSO) as a selective GS inhibitor.

For glutamate dehydrogenase activity, frozen forebrain was processed using the Mitochondrial Isolation Kit from Sigma-Aldrich. The final mitochondrial pellet was resuspended in mitochondrial resuspension buffer containing 0.32 M sucrose, 10 mM Tris-HCl pH 7.4, 0.5 mM EDTA, 0.1% Triton X-100, and protein concentration was determined using the Bio-Rad Protein Assay. Glutamate dehydrogenase activity was determined as described previously (81). Briefly, activity in the direction of oxidative deamination was assayed in a UV/Vis spectrophotometer (HP 8452A, GMI-Inc., Ramsey, MN) at 25°C using 15 µg of mitochondrial protein in a final volume of 200 µL of assay buffer (150 mM KCl,

0.1 mM rotenone, 20 mM Tris-HCl pH 7.6, 2 mM EGTA, 1 mM NAD⁺, 2.5 mM glutamate). The velocity of each reaction was calculated from the linear portion of the change in NADH absorbance measured at 340 nm. Enzymatic specificity of the assay was determined using 20 μ M GTP as a selective GDH inhibitor.

For mitochondrial aspartate aminotransferase activity, mitochondrial extracts were prepared exactly as described for GDH activity. Enzymatic activity was determined as described previously (82), with minor modifications. Briefly, activity in the direction of α -ketoglutarate production was assayed spectrophotometrically using 15 μ g of mitochondrial protein in a final volume of 200 μ L of assay buffer (0.02% BSA, 0.1 mM rotenone, 100 mM ADP, 30 mM NH₄Cl, 120 μ M NADPH, 5 mM Glu, 2.5 mM oxaloacetic acid, and 15 μ g of purified bovine GDH). The decrease in NADPH absorbance at 340 nm at room temperature was followed over 15 minutes and used to calculate the velocity of mAspAT activity. Enzymatic specificity of the assay was determined using 1 mM aminooxyacetic acid (AOAA) as a selective mAspAT inhibitor.

For synaptosomal glutamate uptake, crude synaptosomes were prepared from forebrain as previously described (49, 83) with minor modifications. Briefly, animals between 10-12 weeks of age were anesthetized with isoflurane and decapitated. Forebrains from wild-type, *nNOS*^{-/-}, and *eNOS*^{-/-} mice was dissected and homogenized on ice in 20 volumes of sucrose buffer (0.32 M sucrose, 0.1 mM DTPA pH 5.3), while protected from light. Homogenates were centrifuged at 800 x g for 10 minutes at 4°C, and the supernatants were isolated for further processing. Samples from wild-type mice homogenized in sucrose buffer without DTPA were also pre-treated at 4°C with 50 μ M CuSO₄/500 μ M ascorbate for 30 minutes in the dark to remove NO from S-nitrosocysteine. All

samples were then centrifuged at 20,000 x g for 20 minutes at 4 °C, and the pellets were resuspended in 0.32 M sucrose, 0.1 mM DTPA (pH 5.3). Samples were then centrifuged a final time at 4 °C at 20,000 x g for 20 minutes, with pellets finally resuspended in 0.32 M sucrose (pH 5.3). Sodium-dependent transport of L-³[H]glutamate was then measured as described previously (49, 83) in the presence or absence of 0.3 mM dihydrokainate (DHK) in order to determine DHK-sensitive glutamate uptake (as a measure of excitatory amino acid transporter 2 activity).

Plasmid Constructs

Plasmid expressing rat excitatory amino acid transporter 2 was obtained from Dr. Baruch Kanner (Hebrew University, Hadassah Medical School, Jerusalem, Israel). The Quikchange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) was used to introduce single amino acid mutations in the transporter as follows. Cysteine-to-serine mutations were introduced using the forward/reverse primer pairs: for Cys³⁷³, 5'-TTGCCTGTCACCTTCCGTAGCTTGAAGATAATC-3' and 5'-GATTATCTTCCAAGCTACGGAAGGTGACAGGCAA-3'; and for Cys⁵⁶², 5'-ATGGAAAGTCAGCTGACAGCAGTGTTGAGGAAGAA-3' and 5'-TTCTTCCTCAACACTGCTGTCAGCTGACTTTCCAT-3', resulting in the C373S/C562S construct. All plasmid constructs were verified by sequencing prior to use.

Cell Culture, Transfection, and CysNO Treatment

HEK-293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with Glutamax from Life Technologies (Carlsbad, CA) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 ng/mL streptomycin, at

37°C in air with 5% CO₂. Cells were plated at a density of 1.25×10^5 cells/cm² and cultured under normal conditions for 24 hours. Cells were transfected with wild-type excitatory amino acid transporter 2 (WT GLT1) or the double mutant C373S/C562S transporter using the Clontech CalPhos transfection kit (Clontech, Mountain View, CA) according to manufacturer's protocol. At eighteen hours post-transfection the growth medium was replaced with serum-free DMEM and equilibrated for 2 hours at 37°C. Afterwards, freshly prepared S-nitrosocysteine (CysNO) or cysteine (Cys) was added up to a final concentration of 0.4 mM, and cells were incubated for 2 hours. Cells were extensively washed with PBS, and used for measuring glutamate uptake as described previously (84). Lysates were assayed for protein concentration using the Lowry assay and used to determine excitatory amino acid transporter 2-mediated glutamate uptake initial velocity, described in nmol/mg/min. For cell surface biotinylation studies, cells were processed as described previously (84). Briefly, after Cys or CysNO treatment cell monolayers were washed twice with ice-cold DPBS (pH 7.4) containing 0.1 mM CaCl₂ and 1.0 mM MgCl₂. Cells were then incubated in 2 mL of biotinylation solution (1 mg/mL EZLink Sulfo-NHS-Biotin in DPBS with 0.1 mM CaCl₂ and 1.0 mM MgCl₂) for 30 minutes at 4°C. The biotinylation solution was then aspirated, and excess biotin was quenched by incubating cells with DPBS containing 100 mM glycine for 30 minutes at 4°C. Cells were then lysed in 1 mL RIPA buffer with protease inhibitors (1 µg/mL leupeptin, 250 µM phenylmethanesulfonyl fluoride, 1 µg/mL aprotinin, and 1 mM iodoacetamide). Lysates were then centrifuged to remove cellular debris, and biotinylated proteins were batch extracted from the supernatant using UltraLink monomeric avidin beads. LDS sample buffer was added to fractions containing total cell lysate,

biotinylated proteins (cell surface proteins), and nonbiotinylated proteins (intracellular proteins). Each fraction was diluted and loaded such that the sum of immunoreactivity in the nonbiotinylated and biotinylated fractions should equal the immunoreactivity found in the lysate, assuming avidin extraction of biotinylated proteins is 100%. For reversibility studies, uptake was studied in cells at 0, 60, 120 and 240 minutes after the removal of CysNO. Additionally, lysates from each treatment condition were processed in parallel as previously described (28-30) for S-nitrosoprotein enrichment and subsequent Western blot detection of S-nitrosylated excitatory amino acid transporter 2.

Molecular Modeling of the Effects of S-nitrosylation

The 2.4Å resolution crystal structure of mitochondrial aspartate aminotransferase was taken from PDB 3PD6 (53). The 2.7Å resolution crystal structure of human glutamate dehydrogenase was taken from PDB 1L1F (54). The 2.6Å resolution crystal structure of human glutamine synthetase was taken from PDB 2QC8 (55). Structures of mAspAT with S-nitrosyl modified cysteine (SNC) at positions 106 and 295, GDH with SNC at position 59 (equivalent to position 112 in the mouse sequence) and GS with SNC at positions 99, 183, 269 and 346 were built using VMD and a custom SNC residue template (56). Electrostatic potentials from the cysteine or SNC residues were calculated using the finite difference Poisson-Boltzmann method as described previously (57-58). The solvent was assigned a dielectric of 78.6 and an ionic strength of 145 mM. The protein was assigned a dielectric of 2. Partial atomic charges for the unmodified cysteine residue were taken from the CHARMM27 molecular mechanics forcefield (59). Atomic charges for SNC were obtained from electronegativity neutralization using the program QEQUIL (60), giving +0.35

(C β H₂), -0.17 (S γ), +0.06 (N δ), and -0.24 (O ϵ), in atomic charge units. Structures and potentials were visualized using PyMol (61).

Statistical analysis

Data were analyzed with GraphPad Prism 5.0d software. All normally distributed data were displayed as means \pm SE. Groups were analyzed by one- or two-way analysis of variance (ANOVA), with appropriate post-hoc tests.

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CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Summary

S-nitrosylation has gained prominence in recent years as a major mechanism for the NO -dependent regulation of structure and function for individual proteins. However, much is still unknown regarding how this posttranslational modification may coordinate diverse biological processes in response to specific stimuli. For this thesis, we uncovered multiple protein targets of physiological S-nitrosylation in the mouse that rely on either nNOS-derived or eNOS-derived NO for detection, and demonstrate a role for nNOS in the regulation of the glutamate/glutamine cycle during excitatory neurotransmission. In the process, we took advantage of the plethora of data available from our proteomic studies to generate hypotheses that were tested using a variety of metabolomic, biochemical, and molecular biology approaches.

4.2 The necessity of mass spectrometry-based proteomic approaches in comprehensively studying biological change

Proteomic methodologies represent a powerful addition to other global interrogations of cellular response, including genomic and transcriptomic screens. In particular, their ability to identify proteins from complex mixtures (when coupled to upstream chromatographic separation techniques) makes MS-based proteomics a toolset of choice for many cell signaling studies. The value of this work can be further enhanced with the use of various complementary approaches, including stable isotopic labeling-based protein quantification and/or selective enrichment techniques available for specific posttranslational

modifications. Despite these advances, the depth of proteomic analysis is still limited in comparison to published genomic screens: in humans, only ~84% of protein-coding genes identified in the genome have been matched to proteins detected by MS¹. This gap will narrow in the coming years, however, with refinements in both genome annotation and proteomic methodologies.

Regardless of this caveat, MS-based proteome analysis still represents the most useful route for investigating global changes in protein homeostasis that accompany given physiological stimuli, and can generate the preliminary information needed for more targeted hypotheses of biological function.

Application of our protocol for S-nitrosoprotein enrichment and subsequent LC-MS/MS analysis to the wild-type mouse brain yielded the most comprehensive physiological S-nitrosoproteome currently available for this organ, with an improvement of 225 additional sites and 92 additional protein targets over previous reports. Comparison of this dataset to those we obtained from nNOS^{-/-} and eNOS^{-/-} brain allowed us to make hypotheses about the contributions of nNOS-derived [•]NO to normal CNS function, and afforded the opportunity to explore them in more detail using traditional biochemistry and molecular biology approaches. In doing so, we provided evidence for S-nitrosylation as a global regulator of synaptic transmission through its regulation of glutamate availability, and offered a prime example for the importance of MS-based proteomics in leading to focused mechanistic studies.

4.3 Systemic regulation of brain physiology by S-nitrosylation

Our work uncovered 136 protein targets of S-nitrosylation in the wild-type mouse brain. Subsequent gene ontology analysis revealed 6 of these proteins to be directly involved in glutamate/GABA metabolism and the glutamate/glutamine cycle, with 4 proteins further scrutinized for S-nitrosylation-dependent regulation of glutamate oxidation/transport/recycling to glutamine. Intriguingly, we also found an additional 29 proteins that participate in various stages of cellular metabolism, including glycolysis (Figure 1), the tricarboxylic acid (TCA) cycle (Figure 2), and oxidative phosphorylation (Table 1). Each of these processes indirectly contributes to steady-state levels of glutamate, principally through regulation of the synthesis or consumption of its 5-carbon backbone. Curiously, some proteins from each pathway were found to be S-nitrosylated in WT, eNOS^{-/-}, and nNOS^{-/-} brain, suggesting one or both of the following possibilities: 1) redundancy between eNOS- and nNOS-derived \cdot NO in modifying said protein(s), or 2) \cdot NO from another source common to all genotypes (such as iNOS) was utilized instead. Conversely, a few proteins from each pathway were only observed in the WT brain, suggesting that for them both eNOS- and nNOS-derived \cdot NO may be necessary to modify enough protein to be detected by our method. Regardless, given the significant convergence between S-nitrosylation, metabolism, and excitatory neurotransmission, a role for eNOS- and/or nNOS-derived \cdot NO in coordinating the latter two processes is a strong possibility.

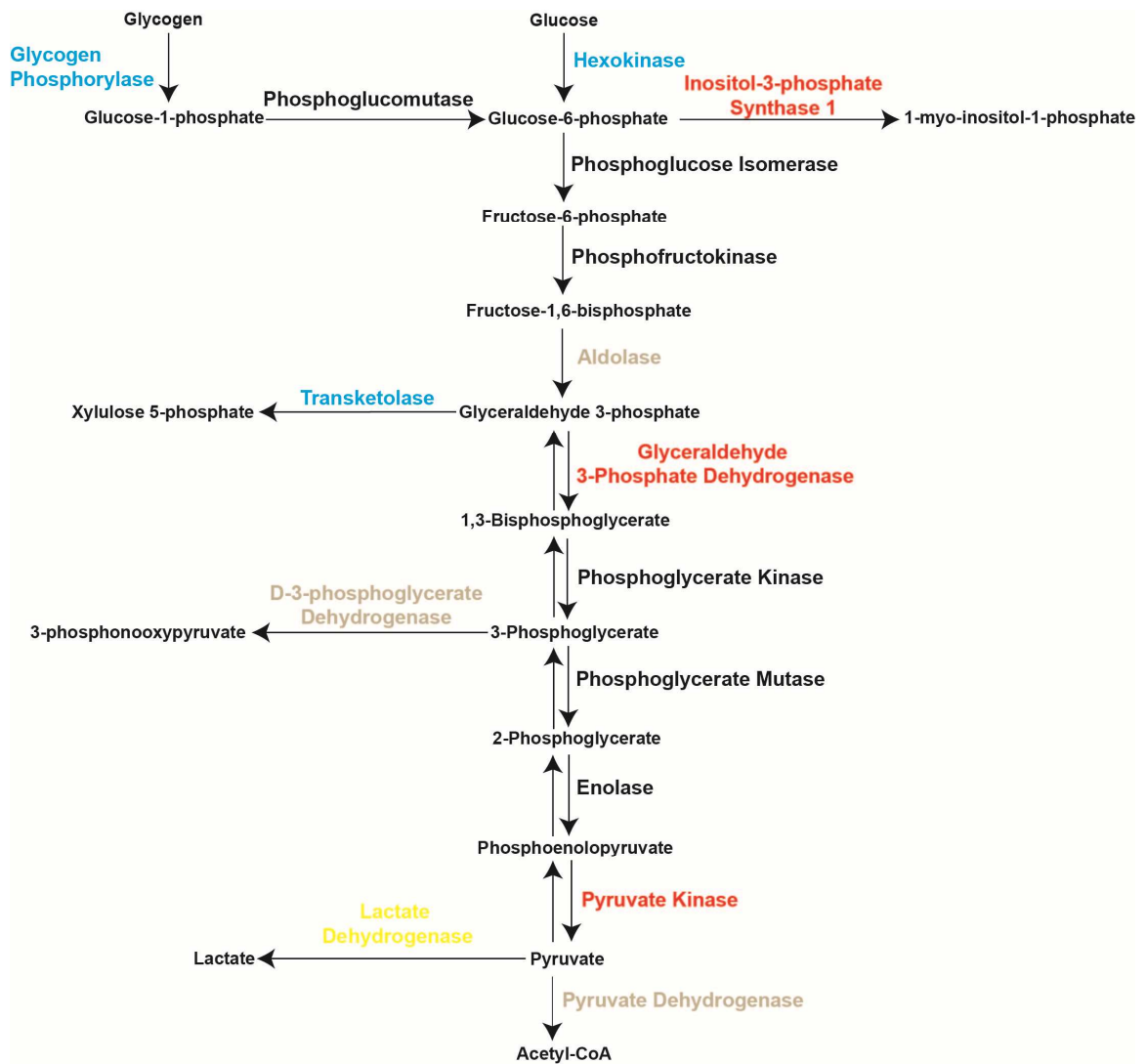


Figure 4.1: S-Nitrosylation in Glycolysis. Multiple enzymes involved in the synthesis and metabolism of glycolytic substrates are modified by cysteine S-nitrosylation. 1) Enzymes denoted in blue had detectable S-nitrosocysteine residues in wild-type and nNOS^{-/-} brain, but not eNOS^{-/-} (eNOS-dependent). 2) Those denoted in red had detectable S-nitrosocysteine residues in wild-type and eNOS^{-/-} brain, but not nNOS^{-/-} (nNOS-dependent). 3) Those denoted in green had detectable S-nitrosocysteines in wild-type, nNOS^{-/-}, and eNOS^{-/-} brain (suggesting redundancy between nNOS and eNOS, or possibly iNOS dependence). 4) Those denoted in yellow were only found to be modified in wild-type brain, not nNOS^{-/-} or eNOS^{-/-} (require both isoforms). 5) Those denoted in black were not detectable as S-nitrosylated in any genotype.

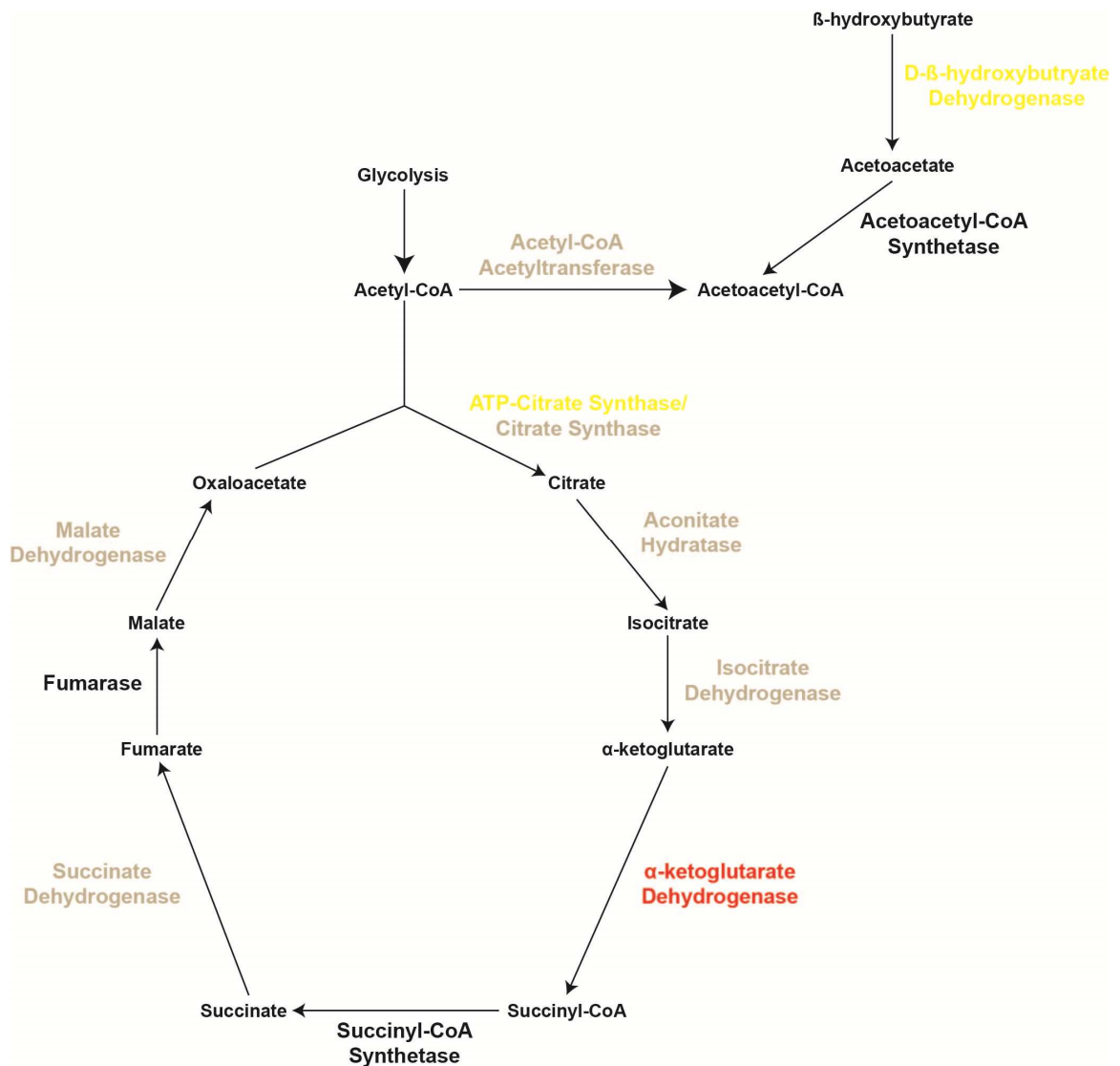


Figure 4.2: S-Nitrosylation in the Tricarboxylic Acid Cycle. Several enzymes in the TCA cycle are modified by cysteine S-nitrosylation. **1)** Enzymes denoted in blue had detectable S-nitrosocysteine residues in wild-type and nNOS^{-/-} brain, but not eNOS^{-/-} (eNOS-dependent). **2)** Those denoted in red had detectable S-nitrosocysteine residues in wild-type and eNOS^{-/-} brain, but not nNOS^{-/-} (nNOS-dependent). **3)** Those denoted in green had detectable S-nitrosocysteines in wild-type, nNOS^{-/-}, and eNOS^{-/-} brain. **4)** Those denoted in yellow were only found to be modified in wild-type brain, not nNOS^{-/-} or eNOS^{-/-} (suggesting redundancy between nNOS and eNOS, or possibly iNOS-dependent). **5)** Those denoted in black were not detectable as S-nitrosylated in any genotype.

Protein Name	Uniprot ID	WT	eNOS^{-/-}	nNOS^{-/-}
NADH dehydrogenase [ubiquinone] iron-sulfur protein 6, mitochondrial (Complex I)	P52503	X	X	X
Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial (Complex II)	Q8K2B3	X	X	X
Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial (Complex II)	Q921G7	X		
Cytochrome b-c1 complex subunit 6, mitochondrial* (Complex III)	P99028	X	X	
Cytochrome c1, heme protein, mitochondrial (Complex III)	Q9D0M3	X		
Cytochrome c oxidase subunit 5B, mitochondrial* (Complex IV)	P19536	X	X	X
ADP/ATP translocase 1	P48962	X		
ATP synthase subunit alpha, mitochondrial	Q03265	X		
ATP synthase subunit gamma, mitochondrial	Q91VR2	X	X	

Table 4.1: S-Nitrosylated Proteins in Oxidative Phosphorylation. At least one protein in each of the 4 major complexes of the electron transport chain contains S-nitrosocysteines that were detected in the wild-type mouse brain. S-nitrosoprotein targets are denoted above, along with the genotypes in which they were observed.

Many of the emergent concepts of NO -dependent metabolic control found in our work are supported by previous reports. eNOS-derived NO from endothelial cells has been shown to increase aerobic glycolysis and lactate production in astrocytes in co-cultures via HIF-1 α -mediated transcription², a result reinforced by earlier studies documenting elevated astrocytic glycolysis after treatment with NO donors³⁻⁴. Such effects on glucose metabolism may at least in part be explained by our proteomic analyses, which found eNOS-dependent S-nitrosylation of enzymes responsible for the first steps of glucose phosphorylation (Figure 1). Our identification of nNOS-dependent S-nitrosylation of inositol-3-phosphate synthase 1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the brain (Figure 1) also suggests a role for nNOS-derived NO in the regulation of glycolysis, with nNOS-dependent GAPDH S-nitrosylation validated by previous studies⁵⁻⁶. Additionally, NMDA receptor-dependent activation of nNOS has been associated with inhibition of the TCA cycle in rat striatum⁷, an observation consistent with both the increased glutamate oxidation⁸ and reduced numbers of S-nitrosocysteine residues in TCA cycle enzymes we identified in nNOS $^{-/-}$ brain (Figure 2). However, though differences exist between eNOS-derived and nNOS-derived S-nitrosylation in their respective protein targets, it may very be that both NOS isoforms are used to coordinate the large-scale shifts in metabolism necessary to support changes in synaptic plasticity.

Taking into account the intimate links that we and others have established between the glutamate/glutamine cycle⁸, glucose metabolism⁹, and synaptic plasticity¹⁰, one can appreciate the metabolic support necessary for long-term changes in excitatory neurotransmission. Based on the data presented herein, nitric oxide may act as a mediator between these biological processes via specific NOS isoform-dependent S-nitrosylation of key effector proteins in each cellular pathway. Moreover, in lieu of the regulatory contributions of this posttranslational modification to glutamate availability, the developmental significance of nNOS-derived $\cdot\text{NO}$ ¹¹⁻¹⁶ can be better understood.

4.4 Implications and future directions

The results of this study, as well as those of other groups¹⁷⁻²¹, reinforce the vital influence of nNOS-derived $\cdot\text{NO}$ in glutamatergic transmission. Our data suggests that at least part of this $\cdot\text{NO}$ -dependent regulation is achieved through the S-nitrosylation of key proteins involved in glutamate transport, oxidation, and recycling to glutamine. Still, the relevance of this phenomenon to multiple aspects of neurobiology, including plasticity, development and neurodegeneration, is yet to be explored.

4.4.1 Stoichiometry

Our findings revealed that roughly 8-25% of all molecules for each of the four proteins studied (GLT1, GDH, mAspAT, and GS) were modified by S-nitrosylation⁸, values that are in line with those described for other enzymes²². At

first glance, these numbers appear incongruous with the changes in protein activity observed between wild-type and nNOS^{-/-} brain (or after S-nitrosothiol elimination in wild-type extracts): GLT1, GDH, and mAspAT function was inhibited by 20-80% when S-nitrosylated *in vivo*, while GS activity was decreased by 40% upon S-nitrosothiol elimination⁸. However, each of the four proteins in question assemble into multimeric complexes: GLT1 as a trimer²³, GDH as a hexamer²⁴, mAspAT as a tetramer²⁵, and GS as a decamer²⁶. Given this information, S-nitrosylation of one or more monomers in each multimer may act in a dominant-negative (for GLT1, GDH, and mAspAT) or dominant-positive manner (for GS) in regulating protein function. Such a concept is further reinforced by our molecular modeling studies of the modification for GDH, mAspAT, and GS, which revealed that S-nitrosylation of the cysteines identified *in vivo* generates a repulsive electrostatic wedge between subunits that could plausibly drive allosteric regulation of protein function⁸.

Stoichiometric studies of posttranslational modifications traditionally rely on the substitution of modified amino acids in proteins with “mimetics,” such as aspartate/glutamate (for phosphoserine/phosphothreonine²⁷) or glutamine (for acetyllysine²⁸). On the other hand, such an approach has not been as robustly validated for protein S-nitrosocysteine residues: several groups have attempted to do so through replacement of cysteine residues with alanine²⁹ or tryptophan³⁰ in order to recapitulate the effects of S-nitrosylation, with mixed results. Still, coexpression of “nitrosomimetic” Cys-to-Ala/Cys-to-Trp mutant proteins with non-

nitrosylatable Cys-to-Ser mutants (which, at least for GLT1, have been shown to have comparable basal activity to the wild-type protein⁸) may be worth investigating in order to ask how the stoichiometry of S-nitrosylation relates to its effects on protein function *in vitro* and *in vivo*.

4.4.2 Spatiotemporal Regulation of Nitric Oxide and S-Nitrosylation in Synaptic Plasticity

Most of our analyses of S-nitrosylation and its effects on protein function were performed in samples from 8-12 week old mice of WT, nNOS^{-/-}, and eNOS^{-/-} genotypes. In doing so, we measured enzyme and transporter activity in the presence of steady-state levels of S-nitrosylation established during CNS development in these animals. Because of this, it is natural to wonder about the on/off kinetics of the modification and its effects on glutamate/glutamine metabolism, particularly within time scales used for monitoring acute changes in synaptic function (milliseconds to seconds) and synaptic plasticity (minutes to hours). Unfortunately, it is not currently possible to detect changes in protein S-nitrosylation within the short periods of time used for measuring acute changes in synaptic function. Despite this limitation, however, conclusions can still be made about the roles of [•]NO and S-nitrosylation in supporting synaptic plasticity.

Several long-term changes in plasticity (i.e. in long-term potentiation) occur minutes to hours after the initial paired presynaptic/postsynaptic tetanic stimuli³¹. [•]NO-dependent signaling is thought to augment this process, rather

than acting alone as a retrograde neurotransmitter³². Moreover, within a similar period of time following stimulation, NO diffuses over a radius of 100-200 μm from its postsynaptic source^{19,33}, allowing it to reach multiple cellular compartments of the tripartite synapse. Given that our analyses⁸ of glutamine/glutamate utilization (via metabolomics) and S-nitrosylation reversibility (*in vitro*) was performed within similarly relevant time periods (minutes to hours following chemical stimulation or S-nitrosocysteine treatment), the data we obtained points toward S-nitrosylation as more of a long-term regulator of synaptic plasticity rather than a short-term effector of synaptic function. This finding is reinforced by previous reports from other groups, which have documented differences in S-nitrosylation and function of specific proteins within a period of minutes to hours following stimulation³⁴⁻³⁶.

4.4.3 Therapeutic Implications in Neurological Disorders

Neuronal nitric oxide participates in several processes during CNS maturation, including neurogenesis³⁷⁻⁴¹, excitatory synapse development⁴²⁻⁴³, neuronal migration⁴⁴ and differentiation³⁷. In light of the reciprocity between glutamate metabolism and synaptic activity¹⁰, as well as our own data, it may be that nNOS-derived protein S-nitrosylation contributes to the synapse-pruning events underlying the developmental transition between the juvenile and adult brain⁴⁵⁻⁴⁶. Mechanistically, this may be accomplished by facilitating spatially restricted elevations of extracellular glutamate to strengthen specific synapses, while weakening others by allowing astrocytic glutamate removal and

subsequent oxidation. Additionally, alterations in these events could play a role in aberrant neurological disorders such as schizophrenia, autism spectrum disorder (ASD), and epilepsy due to changes in nNOS expression and its associated synthesis of $\cdot\text{NO}$ ⁴⁷⁻⁵¹. The study of these processes in a developmental context is worthy of further inquiry, and may lead to novel therapeutic avenues for specific diseases in the CNS.

Several forms of neurodegeneration exhibit evidence of glutamate-dependent excitotoxicity, including Alzheimer's disease⁵² (AD), Parkinson's disease⁵³ (PD), stroke⁵⁴, and traumatic brain injury⁵⁵ (TBI). One common component of pathological progression in these diseases is the increased synthesis of $\cdot\text{NO}$ by nNOS, which in some cases is subsequently followed by the S-nitrosylation of specific proteins that contribute to cell death⁵⁶. In particular, genetic deletion or inhibition of nNOS in rodents is neuroprotective following cerebral ischemia, MPTP treatment, or TBI^{11,57-61}. Our results may provide an explanation for this effect, by implicating nNOS-derived protein S-nitrosylation in generating the excessive synaptic glutamate concentrations necessary for downstream excitotoxic events. If this is the case, one way to potentially ameliorate these deleterious effects is to administer branched-chain amino acids (BCAAs): these supplements can allosterically activate glutamate dehydrogenase⁶², leading to increased glutamate oxidation and, as a corollary, decreased glutamate availability for excitotoxic processes. BCAA administration has already been shown promote cognitive rehabilitation after TBI in rodents⁶³⁻⁶⁴,

and may promote a similar recovery following ischemia or other forms of neurodegeneration. However, the perturbation of this pathway under such conditions needs to be confirmed before alternative therapeutic options are considered.

The data presented in this work provides compelling evidence for nNOS-derived protein S-nitrosylation in the regulation of synaptic glutamate levels during normal function in the CNS. By extending its findings to additional physiological and non-physiological paradigms, we can ask more detailed questions about the functional relevance in synaptic activity, as well as whether aberrant NO-dependent regulation of glutamate metabolism plays a role in the pathological progression of different neurological disorders. Furthermore, in doing so we can explore opportunities for therapeutic intervention that focus on either nNOS (as has been done for ischemia⁶⁵) or specific steps of glutamate/glutamine metabolism.

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